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Signaling Pathways of Heme Oxygenase-1 Gene Activation by Lipopolysaccharide and NAD(P)H Oxidase Inhibitor 4-(2-Aminoethyl) Benzenesulfonyl Fluoride in Monocytes

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Inaugural-Dissertation
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ABBREVIATIONS

AEBSA	4-(2-aminoethyl) benzenesulfonamide
AEBSF	4-(2-aminoethyl) benzenesulfonyl fluoride
AP-1	activator protein-1
CO	carbon monoxide
COX-2	cyclooxygenase-2
CRE	cAMP RE
DMEM	Dulbecco's modified eagle's medium
ERK	extracellular signal-regulated kinase
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HO	heme oxygenase
IKKβ	IκB kinase β
IκB	inhibitor of NF-κB
iNOS	inducible nitric oxide synthase
JNK	c-jun N-terminal kinase
LPS	lipopolysaccharide
MAPKs	mitogen-activated protein kinases
NF-κB	nuclear factor-κB
NO	nitric oxide
Nrf2	nuclear factor-erythroid2 (NF-E2) related factor
PBMC	peripheral blood monocytes
PI3K	phoshatidyl-inositol 3-kinase
PKA	cAMP-dependent protein kinase
PKB	protein kinase B
PKC	protein kinase C
PKG	cGMP-dependent protein kinase
RE	regulatory element
ROS	reactive oxygen species
RT	room temperature
SDS	sodium dodecyl sulfate
TF	transcription factor
TNFα	tumor necrosis factor-α
TPA	12-*O*-tetradecanoylphorbol 13-acetate
USF	upstream stimulatory factor
w/v	weight per volume

CONTENTS

1. INTRODUCTION

1.1. Heme oxygenase (HO)

Heme oxygenase (HO) (EC 1.14.99.3) catalyzes the first and rate-limiting step of heme degradation. HO breaks up the heme tetrapyrrole ring to yield equimolar amounts of biliverdin, carbon monoxide (CO) and iron (Fig. 1). Biliverdin, in turn, is converted into bilirubin by biliverdin reductase in a non-rate-limiting enzyme reaction (Maines, 1997).

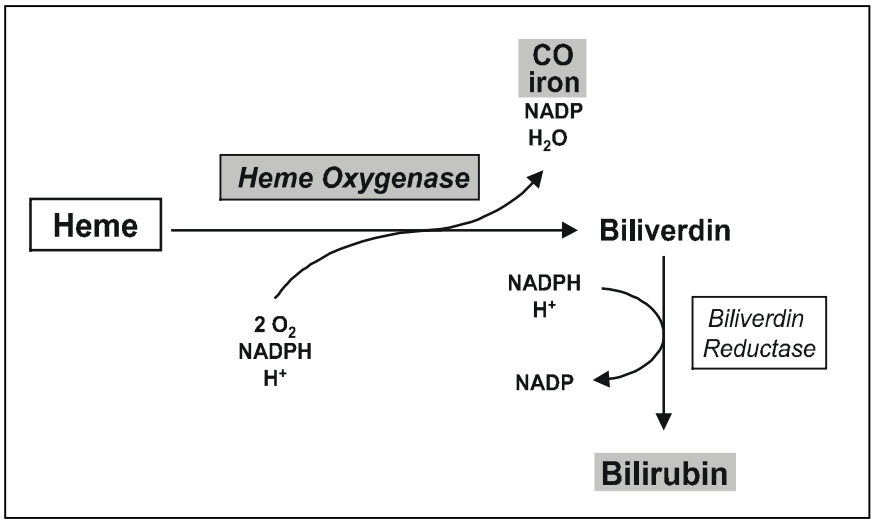

Fig. 1. The heme oxygenase enzyme reaction. Scheme of catalytic conversion of heme into bilirubin, CO and iron.

1.1.1. Isoforms of HO

HO-1 was initially described in 1968 by Tenhunen and colleagues (Tenhunen et al., 1968) and a second isoform of HO, designated HO-2, was identified in 1986 (Maines et al., 1986). The HO-1 and HO-2 genes have about 43% nucleotide sequence similarity. The inducible HO isozyme, HO-1, also known as 32-kDa heat-shock protein, exhibits low basal gene expression levels in most cells and tissues. High gene expression levels of HO-1, are detected in spleen and liver tissue macrophages (Kupffer cells) in which senescent erythrocytes are sequestered and destroyed. By contrast, the non-inducible HO isoform HO-2, exhibits high constitutive gene expression preferentially in brain and testis (Maines, 1988; Otterbein and Choi, 2000). The 36-kDa protein HO-2 is considered a sensor of intracellular heme levels (Wagener et al., 1999) (see also table 1). HO-1 and HO-2 are products of distinct genes. Both HO isoforms are highly conserved throughout evolution and are found in

a wide range of organisms such as bacteria, fungi, plants and mammalians. Sequence homology between the mammalian rat, mouse and human genes is higher than 80% or 90% for HO-1 and HO-2, respectively (Maines, 1997). The significance of a third isozyme of HO (HO-3) which has been described by the group of Maines is unclear (McCoubrey et al., 1997).

Table 1. Characteristics of HO-1 and HO-2.

	HO-1	HO-2
Major function	Catalytic heme degradation	Intracellular heme sensor (?)
Inducibility	Highly inducible by many stimuli	Glucocorticoid
Localization	Ubiquitous, highest in spleen, liver and kidney	Mainly in brain and testis
Molecular mass (kD)	≈ 32	≈ 36

1.1.2. The inducible isoform HO-1

HO-1 gene expression is induced not only by its substrate heme or heme-containing compounds, but also by non-heme containing compounds (Table 2) that increase the cellular production of reactive oxygen species (ROS), such as hydrogen peroxide, UV light, endotoxin, heavy metals and sodium arsenite (Maines, 1988; Otterbein and Choi, 2000). Due to this large array of stress stimuli that induce HO-1 gene expression, HO-1 has been considered for many years to serve cytoprotective functions against oxidative stress.

1.1.3. Functional significance of the HO enzyme reaction

The HO enzyme reaction is of physiological significance because HO not only degrades the prooxidant heme, but also produces biliverdin, CO and iron. These HO products have been recognized to serve regulatory and protective functions (Otterbein et al., 2003).

1.1.3.1. The tetrapyrrole heme

Heme (Fe-protoporphyrin IX) is a tetrapyrrole with a central iron ion (Fe^{2+}) that is non-covalently linked to the four ligand groups of porphyrin (Wagener, 2003). Heme is

synthesized via a pathway of eight enzymes and has contradictory biological functions. On the one hand, heme is an essential compound of various hemoproteins that are involved in transport and storage of oxygen (hemoglobin and myoglobin), mitochondrial respiration (cytochromes), drug metabolism (cytochrome P450s), cellular antioxidant defense mechanisms (peroxidases and catalase) and steroid hormone biosynthesis (Ryter and Tyrrell, 2000; Wijayanti et al., 2004). In addition, heme is a crucial component of several major enzymatic systems such as inducible nitric oxide (NO) synthase (NOS), cyclooxygenase-2 (COX-2) and soluble guanylate cyclase (Maines, 1997; Wijayanti et al., 2004). On the other hand, excess heme can be severely toxic in its 'free', non-protein bound, form, specifically during pathophysiological conditions such as hemolysis or rhabdomyolysis. 'Free' heme can generate ROS which, in turn, may cause DNA damage, lipid peroxidation and protein denaturation (Muller-Eberhard and Fraig, 1993; Ryter and Tyrrell, 2000). Therefore, the biological activity of heme needs to be controlled tightly by enzymatic heme synthesis and/ or degradation (Wijayanti et al., 2004).

Table 2 . Conditions and nonheme compounds that induce HO-1 .

Condition	References
Cytokines	Terry et al.,1999
Endotoxin	Camhi et al., 1995
Heavy metals	Elbirt et al., 1998
Heat shock	Okinaga and Shibahara, 1993
Hydrogen peroxide	Keyse and Tyrrell, 1989
Hyperoxia	Otterbein et al., 1999
Hypoxia	Motterlini et al., 2000
Nitric oxide	Willis et al., 1995
Phorbol esters	Alam and Den, 1992
Prostaglandin	Koizumi et al., 1995
Shear stress	Wagner et al., 1997
Sodium arsenite	Elbirt et al., 1998
Thiol scavengers (GSH depletion)	Ewing and Maines, 1993
Ultraviolet A	Keyse and Tyrrell, 1989

1.1.3.2. The cytoprotective antioxidant bilirubin

The HO product biliverdin is rapidly converted into bilirubin by biliverdin reductase in mammalian cells. It has been shown by Stocker and colleagues that the bile pigment bilirubin has antioxidant properties (Stocker et al., 1987). This initial finding has been confirmed by others who demonstrated that HO-derived bilirubin provides cellular protection to neuronal cells (Dore et al., 1999). A comprehensive overview on the protective effects of bilirubin has recently been given by Kapitulnik (2004).

1.1.3.3. The second messenger gas CO

The toxic effects of CO have been known for many years. Since CO binds hemoglobin with higher affinity than O_2, O_2 delivery to tissues and organs is blocked by this gaseous molecule. More recently, HO-derived CO has been recognized to be an important cellular messenger with various physiological functions similar to those of the signaling gas NO. In contrast to NO, however, which forms the toxic peroxynitrite with superoxide, CO does not form radicals. CO is involved in the regulation of hepatobiliary functions such as cytochrome P450-dependent biotransformation, and HO-1-derived CO has been shown to protect the hepatic microcirculation under stress conditions (Suematsu and Ishimura, 2000). Furthermore, CO is involved in the regulation of the cardiovascular tone (Maines, 1997; Sammut et al., 1998; Otterbein and Choi, 2000; Otterbein et al., 2003).

1.1.3.4. Iron

Enzymatic degradation of heme by HO produces iron which is an essential cofactor of numerous cellular enzymes and redox-dependent proteins. Similar to ‘free’ heme, excess iron is cytotoxic by producing ROS via the Fenton reaction and needs to be contained by an iron-binding protein (Ponka, 1997). In accordance with this concept synthesis of the iron-binding protein ferritin has been demonstrated to be induced in coordination with HO-1 activity during the cellular stress response of skin fibroblast (Vile and Tyrrell, 1993; Ryter and Tyrrell, 2000). This coordinate induction of HO-1 and ferritin is assumed to prevent iron-mediated cytotoxicity. The regulatory role of HO-1 for iron homeostasis is also supported by the observation that genetic HO-1 deficiency causes an atypical iron deficiency anemia and a simultaneous iron overload of the liver (Poss and Tonegawa, 1997). Moreover, it has been demonstrated that cytoprotection of cell cultures by HO-1 can be attributed to augmented cellular iron efflux which is regulated by an iron ATPase (Ferris et al., 1999; Barañano et al., 2000).

1.2. Phenotypical alterations in genetic HO-1 deficiency

The physiological significance of HO-1 has clearly been demonstrated in a HO-1 knockout mouse model and a case of human genetic HO-1 deficiency. In HO-1 knockout mice Poss and Tonegawa (1997) have demonstrated that these animals exhibit growth retardation, signs of a normochromic, microcytic anemia and iron overload of the kidney and liver. Moreover, HO-1 deficient mice have a proinflammatory phenotype with an exaggerated activation of mononuclear phagocytes and are highly susceptible to oxidative stress as indicated by a higher mortality of these animals in response to treatment with endotoxin. These findings from HO-1 knockout mice were essentially confirmed in a case of human genetic HO-1 deficiency (Yachie et al., 1999) in which similar phenotypical alterations were observed (see Table 3). Sequence analysis of this patient's HO-1 gene revealed complete loss of exon 2 of the maternal allele and a two-nucleotide deletion within exon 3 of the paternal allele (Yachie et al., 1999).

Table 3. Phenotypical alterations in human and mouse genetic HO-1 deficiency (Yachie et al., 1999).

Findings	Human	Mouse
Intrauterine death	Stillbirth, abortion	20% birth rate
Growth failure	+	+
Anemia	+	+
Fragmentation	+	?
Iron binding capacity	Increased	Increased
Ferritin	Elevated	Elevated
Iron deposition	+	+
Hepatomegaly	+	+
Splenomegaly	Asplenia	+
Lymph node swelling	+	+
Leukocytosis	+	+
Thrombocytosis	+	?
Coagulation abnormality	+	?
Endothelial injury	+	?
Hyperlipidemia	+	?

1.3. Transcriptional regulation and signal transduction pathways of HO-1

Stimulation of the HO-1 gene by most, if not all, stimuli is primarily controlled at the transcriptional level (Choi and Alam 1996) and a variety of regulatory elements (RE) and transcription factors (TF) have been demonstrated to be involved in this regulation. The broad spectrum and chemical diversity of reagents that induce HO-1 suggests that various signaling pathways are involved in the regulation of this gene such as mitogen-activated protein kinases (MAPK), protein kinase C (PKC), cAMP-dependent protein kinase A (PKA), or cGMP-dependent protein kinase G (PKG) (Immenschuh and Ramadori, 2000).

1.3.1. Regulatory elements and transcription factors that regulate HO-1 gene expression

Various REs that govern the transcriptional regulation of the HO-1 gene have been identified in the promoter 5'-flanking region of the HO-1 gene (Choi and Alam, 1996). Identification of inducer-dependent REs not only provides important information on the molecular mechanisms that govern HO-1 gene expression, but also on the cellular signaling cascades that are involved in HO-1 gene regulation. The promoter 5'-flanking regions of the human, rat, mouse and chicken HO-1 genes have been analyzed by functional studies in transiently and stably transfected cell cultures and by studies on DNA-protein interactions (Choi and Alam, 1996; Elbirt and Bonkovsky, 1999; Alam and Cook, 2003; Sikorski et al., 2004). Specifically, the rat HO-1 gene including the 5'-flanking gene promoter region has been cloned in 1987 (Müller et al., 1987). In the following, several potential REs have been identified in the proximal 1338 bp of the rat HO-1 gene promoter 5'-flanking region such as a heat shock response element 1 (HSE1) (Okinaga and Shibahara., 1993), HO transcription factor (HOTF) binding element (Sato et al., 1989), prostaglandin J_2 response element (PGJ-2) (Koizumi et al., 1995), cAMP response element/ activator protein-1 binding site (CRE/AP-1) (Immenschuh et al., 1998), E-box element (Kietzmann et al., 2003) and Ets binding site (EBS) (Chung et al., 2005). Moreover, additional potential REs have been identified by sequence comparison such as three copies of the Sp1 binding site, two copies of GCN4, a metal-dependent TF (Müller et al., 1987), an AP-1 site (Sato et al., 1989), a HSE2 (Okinaga and Shibahara., 1993) and a nuclear factor-κB recognition sequence (NF-κB) (Fig. 2).

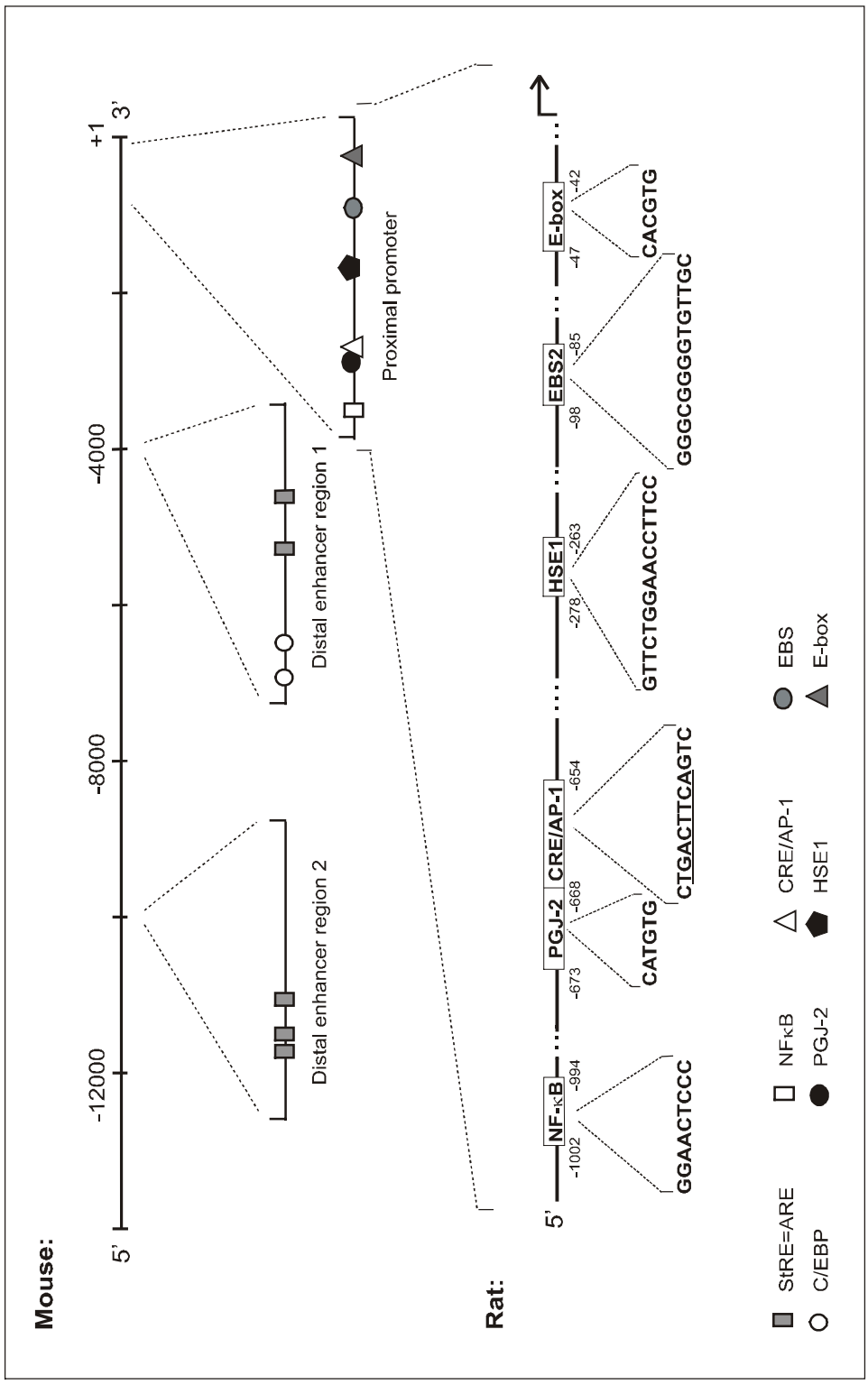

Fig. 2. Scheme of the HO-1 gene promoter. The sequences indicate that mouse and rat HO-1 are almost 100% identical.

While considerable information is available on the functionality of REs of the HO-1 gene, much less is known about the TFs that are involved in this regulation. Several prototypical REs of the mouse HO-1 gene with AP-1 sites that are localized in two far upstream HO-1 promoter regions have initially been shown to mediate the HO-1 stress response (Alam and Den, 1992; Alam, 1994; Alam et al., 1995). Subsequently, these elements have been shown not to be regulated by the TF AP-1, but by NF-E2 related factor E2 (Nrf2) (Alam et al., 1999). Nrf2 belongs to the family of Cap'n'Collar basic leucine zipper proteins and is considered to be a crucial regulator of the cellular stress response (Ishii et al., 2000; Nguyen et al., 2003).

1.3.2. Signaling pathways of HO-1 gene regulation

1.3.2.1. Redox signaling

A large number of studies deals with the regulation of HO-1 gene expression by stimuli that increase the cellular production of ROS such as heme, heavy metals, UV light, hydrogen peroxide, lipopolysaccharide (LPS), or by stimuli that deplete cellular glutathione stores including buthionine sulfoximine, sodium arsenite and iodoacetamide (Applegate et al., 1991; Choi and Alam, 1996). Furthermore, it has been shown that scavengers of ROS such as N-acetyl L-cysteine inhibit or reduce the magnitude of HO-1 induction by oxidative stress (Lautier et al., 1992). These findings indicate that an increase of intracellular ROS and, therefore, the activation of redox-dependent signaling pathways plays a crucial role for the regulation of HO-1 gene expression. Although there are still significant gaps in the understanding of the exact mechanisms of redox signaling, in particular, the intracellular targets of ROS (Finkel, 1998), changes of the cellular redox state modify the activity of specific regulatory protein kinases and protein phosphatases which lead to alterations in the regulation of gene expression (Finkel, 1998).

1.3.2.2. MAP kinases (MAPK)

MAPKs are a family of serine-threonine protein kinases that are activated by a variety of extracellular stimuli and are assumed to play a crucial role for the signal transduction pathways of cellular stress and for the regulation of cell proliferation and differentiation (Chang and Karin, 2001; Kyriakis and Avruch, 2001). HO-1 is induced by numerous stimuli that are also known to enhance the activity of MAPKs. Therefore, it is not surprising that various MAPKs are involved in the activation of HO-1 gene expression. The three major MAPKs (extracellular-regulated kinase (ERK), c-jun N-terminal kinase (JNK) and p38) have

been demonstrated to be involved in HO-1 gene regulation (Elbirt et al., 1998; Immenschuh and Ramadori, 2000).

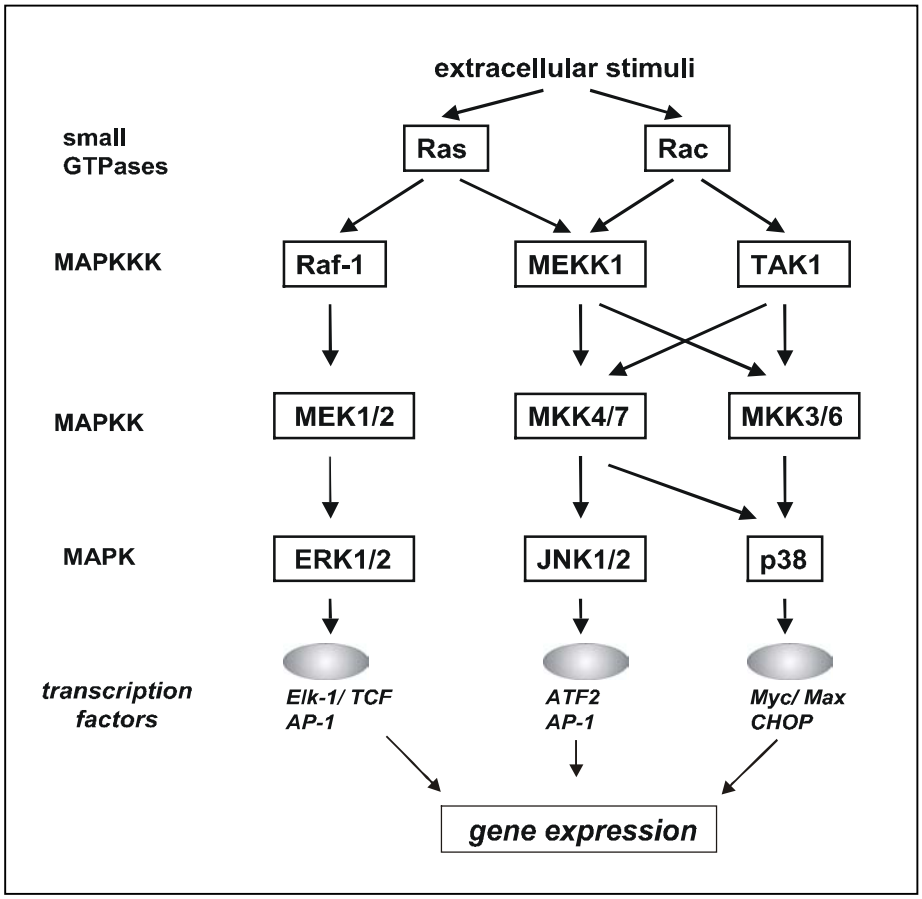

Fig. 3. Scheme of the three major MAPKs signaling pathway

1.3.2.3. Protein kinase C (PKC)

PKC represents a family of related serine-threonine kinases that play an important role for the cellular signal transduction pathways of numerous stimuli (Newton, 1997; Gopalakrishna and Jahen., 2000). The prototypical PKC activator 12-*O*-tetradecanoylphorbol 13-acetate (TPA) induces HO-1 gene expression in various cell culture systems (Alam and Den, 1992; Muraosa and Shibahara, 1993; Alam et al., 1995). TPA-dependent HO-1 gene activation is attenuated by specific inhibitors of PKC, but not by free radical scavengers in fibroblasts indicating that HO-1 induction by TPA is not mediated by the generation of ROS (Alam et al., 1995). It has also been shown that TPA-dependent HO-1 induction is mediated by distinct *cis*-acting REs suggesting that at least two different cell-specific signaling

pathways are involved in this regulatory mechanism (Muraosa and Shibahara, 1993; Alam and Den, 1992). The proinflammatory cytokines tumor necrosis factor (TNFα) and interleukin-1β were also shown to induce HO-1 gene expression via activation of PKC (Terry et al., 1999).

1.3.2.4. cAMP- and cGMP-dependent protein kinases (PKA and PKG)

Intracellular levels of the second messenger cAMP are elevated by a large number of hormones and extracellular stimuli resulting in the activation of PKA. It has been demonstrated that treatment with glucagon and Bt_2cAMP induces HO-1 enzyme activity via activation of the PKA signaling pathway (Bakken et al., 1972; Durante et al., 1997; Immenschuh et al., 1998). By contrast, others have shown Bt_2cAMP inhibit HO-1 enzyme activity in different cells (Sardana et al., 1985). Moreover, an increase of intracellular NO either by NO-releasing agents or via the induction of inducible NO synthase (iNOS) by LPS or cytokines up-regulate HO-1 gene expression in a number of cell lines (Motterlini et al., 1996; Durante et al., 1997; Immenschuh and Ramadori, 1999; Polte et al., 2000). cGMP is known to be the second messenger of at least the short-term actions of NO, which activates the soluble guanylate cyclase. Similar to PKA, cGMP-dependent induction of HO-1 appears to be cell-type specific (Immenschuh et al., 1998).

1.3.2.5. Protein phosphatases (PPs)

A fine-tuned balance between regulatory protein kinases (PKs) and PPs is critical for the control of cellular homeostasis and the important role of PPs for the regulation of gene expression has been demonstrated in various studies (for a review see Hunter, 1995). Okadaic acid, a specific inhibitor of the serine/ threonine PPs 1 and 2A, induced HO-1 transcriptional gene expression via a CRE/AP-1 element that is also responsible for the cAMP- and cGMP-dependent HO-1 induction (Immenschuh et al., 1998; 2000). In addition, specific phosphatases are involved in the transcriptional regulation of HO-1 gene expression by Δ^{12}-prostaglandin J_2 (Koizumi et al., 1995; Negishi et al., 1995).

1.3.2.6. Signaling pathways that suppress HO-1 gene expression

In contrast to the up-regulation of HO-1 gene expression, much less is known about the regulatory mechanisms that suppress HO-1 gene expression. HO-1 gene expression can be down-regulated by angiotensin II in rat vascular smooth muscle cells which is apparently mediated by an increase of intracellular calcium levels (Ishizaka and Griendling, 1997).

Moreover, it has been shown that interferon-γ suppresses HO-1 gene expression in glioblastoma cells (Takahashi et al., 1999). In addition, it has been shown in vascular smooth muscle cells and in rat liver tissue macrophages that LPS-dependent induction of HO-1 is inhibited by the cytokines transforming growth factor-β_1 and interleukin-10 (Pellacani et al., 1998; Immenschuh et al., 1999).

1.4. Regulation of HO-1 gene expression in mononuclear phagocytes - Goal of the study

Mononuclear phagocytes (monocytes, macrophages) are immune cells which play a crucial role for innate immunity. Accumulating evidence suggests that HO-1 expression in mononuclear phagocytes is involved in the regulation of the inflammatory immune response (for a review see Otterbein et al., 2003). The bacterial cell wall component LPS is a prototypical mediator of inflammation and has previously been shown to induce HO-1 in monocytic cells (Camhi et al., 1995; 1998; Immenschuh et al., 1999). LPS binds to the plasma protein LPS-binding protein (LBP) which is recognized by the cell surface receptor CD14, that interacts with the transmembrane Toll-like receptor 4 (TLR4) and its accessory protein MD2 (see Figs. 25, 26 in Discussion). This molecule complex, in turn, initiates the activation of various intracellular signaling cascades in mononuclear phagocytes that cause the generation of ROS and NO as well as the secretion of the proinflammatory cytokines TNFα and interleukin-1 (Watson et al., 1994; Guha and Mackman, 2001).

In mononuclear phagocytes NAD(P)H oxidase is important for the microbicidal mechanisms of these cells and is activated by LPS (Park et al., 2004) and the phorbol ester TPA (Watson et al., 1994; Babior, 1999). Gene expression of NAD(P)H oxidase and HO-1 has been shown to be coordinately increased in monocytes of diabetic patients (Avogaro et al., 2003). Therefore, it was postulated that LPS-dependent HO-1 gene induction would be decreased by the pharmacological NAD(P)H oxidase inhibitor 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF) in RAW264.7 cells. To test this hypothesis, the molecular mechanisms and signaling pathways of HO-1 gene regulation by LPS and the NAD(P)H oxidase inhibitor AEBSF were investigated in RAW264.7 monocytes.

The present study shows that LPS induces the endogenous HO-1 gene expression in RAW264.7 cells on the transcriptional level. Up-regulation of HO-1 promoter activity by LPS is decreased via inhibition of the nuclear factor-κB (NF-κB) signaling pathway by pharmacological NF-κB inhibitors and by overexpression of dominant negative mutants of

NF-κB inducing kinase (NIK), inhibitor of NF-κB (IκB) kinase β (IKKβ), and IκBα. Moreover, the p38 MAPK inihibitor SB203580 and overexpressed dominant negative p38β decreases, whereas p38δ increases, the LPS-dependent induction of HO-1 gene expression. The transcriptional induction of HO-1 gene expression by LPS is mediated by the CRE/AP-site (-668/-654) and an E-box motif (-47/-42) of the proximal rat HO-1 gene promoter region, as indicated by deletion and mutation analysis of luciferase reporter gene constructs.

It is also reported that, unexpectedly, the chemical NAD(P)H oxidase inhibitor AEBSF induces endogenous gene expression and promoter activity of HO-1 in cell cultures of human PBMC and mouse RAW264.7 monocytes. AEBSF-dependent induction of HO-1 gene expression is mediated via activation of the PI3K/PKB signaling pathway as determined by the application of pharmacological inhibitors and overexpression of dominant negative PKB. Similar to the LPS-dependent regulation, AEBSF-dependent induction of HO-1 gene expression is reduced by the p38 inhibitors SB203580 and SB202190 and cotransfection of dominant negative mutants of p38α and p38β, but not that of p38γ and p38δ. AEBSF and PKB-dependent induction of HO-1 promoter activity is markedly reduced by simultaneous mutations of an E-box and the CRE/AP-1 element of the proximal promoter. Finally, the coactivator p300 and the basic helix-loop-helix transcription factor USF2 are shown to be involved in AEBSF-dependent HO-1 gene induction. The data suggest that activation of NF-κB, PKB and p38 MAPK signaling pathways mediate the transcriptional induction of HO-1 gene expression by LPS and AEBSF in monocytes.

2. MATERIALS and METHODS

2.1. Materials

2.1.1. Chemicals and reagents

Acrylamide/bisacrylamide 30%, 37.5:1 (Rotiphorese gel)	Roth, Karlsruhe, Germany
Agarose ultra pure	Roth, Karlsruhe, Germany
Ammonium persulfate (APS)	Serva, Heidelberg, Germany
Ampicillin	Roche, Basel, Switzerland
Bacto agar	Difco, Detroit, MI, USA
Bacto-tryptone	Difco, Detroit, MI, USA
Bromophenol blue	Merck, Darmstadt, Germany
Bovine serum albumin (BSA), fraction V	Serva, Heidelberg, Germany
Cell culture lysis reagent 5x (CCLR)	Promega, Madison, WI, USA
Chloroform	Roth, Karlsruhe, Germany
Dimethylsulfoxid (DMSO)	Sigma-Aldrich, Munich, Germany
Dithiothreitol (DTT)	Sigma-Aldrich, Munich, Germany
Dry milk	Sucofin, Zeven, Germany
Ethanol (100%)	Roth, Karlsruhe, Germany
Ethidium bromide	Dianova, Hamburg, Germany
Ethylenediamine tetraacetic acid (EDTA)	Merck, Darmstadt, Germany
Extract of yeast powder	Merck, Darmstadt, Germany
FuGENE 6 transfection reagent	Roche, Indianapolis, IN, USA
Glycerol	Sigma-Aldrich, Munich, Germany
Glycin	Sigma-Aldrich, Munich, Germany
Glycogen	Roche, Basel, Switzerland
Luciferase assay system	Promega, Madison, WI, USA
Methanol	Roth, Karlsruhe, Germany
Penicillin/Streptomycin	Merck, Darmstadt, Germany
Phenol	Sigma-Aldrich, Munich, Germany
Ponceau S	Serva, Heidelberg,Germany
Potassium acetate	Sigma-Aldrich, Munich, Germany
Protease inhibitor	Roche, Basel, Switzerland
Sodium acetate	Merck, Darmstadt, Germany
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich, Munich, Germany
Tetra-methylethylendiamine (TEMED)	Serva, Heidelberg, Germany
Tris base	Sigma-Aldrich, Munich, Germany
Yeast extract	Difco, Detroit, MI, USA
Tween 20	Sigma-Aldrich, Munich, Germany

All other standard reagents were from Sigma-Aldrich, if not indicated otherwise.

2.1.2. Enzymes

Klenow enzyme	Promega, Madison, WI, USA
T4 DNA ligase	New England Biolabs, Beverly, MA, USA
T4 polynucleotide kinase	Promega, Madison, WI, USA

All restriction enzymes were from New England Biolabs, Beverly, MA, USA

2.1.3. Pharmacological compounds

4-(2-aminoethyl) benzenesulfonamide (AEBSA)	$C_8H_{12}N_2O_2S$	Sigma-Aldrich, Munich, Germany
4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF)	$C_8H_{10}FNO_2S.HCl$	Sigma-Aldrich, Munich, Germany
Caffeic acid phenethyl ester (CAPE)	$C_{17}H_{16}O_4$	Sigma-Aldrich, Munich, Germany
LPS from *E. coli* serotype 0111:B4		Sigma-Aldrich, Munich, Germany
LY294002	$C_{19}H_{17}NO_3$	Calbiochem, La Jolla, CA, USA
Nap-tosyl-L-lysine chloro-methylketone (TLCK)	$C_{14}H_{21}ClN_2\ O_3S.\ HCl$	Calbiochem, La Jolla, CA, USA
PD98059	$C_{16}H_{13}NO_3$	Calbiochem, La Jolla, CA, USA
SB202190	$C_{20}H_{14}FN_3O$	Calbiochem, La Jolla, CA, USA
SB203580	$C_{21}H_{16}N_3OSF$	Calbiochem, La Jolla, CA, USA
SH-5	$C_{29}H_{59}O_{10}P$	Alexis, San Diego, CA, USA
SP600125	$C_{14}H_8N_2O$	Biomol, Hamburg, Germany
TNF-α		Roche, Basel, Switzerland
TPA	$C_{36}H_{56}O_8$	Sigma-Aldrich, Munich, Germany
Wortmannin	$C_{23}H_{24}O_8$	Calbiochem, La Jolla, CA, USA

2.1.4. Markers

Rainbow ^{TM} protein molecular weight	Amersham Biosciences, Freiburg, Germany
Biotin Marker	Cell Signaling, Beverly, MA, USA
DNA molecular weight marker III	Roche, Basel, Switzerland
1 kb ladder	Roche, Basel, Switzerland

2.1.5. Kits

DC Protein Assay kit	Bio-Rad, Munich, German
Compat-able protein assay preparation reagent kit	Pierce, Rockford, IL, USA
ExSite Mutagenesis kit	Stratagene, La Jolla, CA, USA
QIAGEN Plasmid Maxi kits	QIAGEN, Hilden, Germany
QIAGEN Plasmid Midi kits	QIAGEN, Hilden, Germany
QuickChange^{TM} XL Site-Directed Mutagenesis kit	Stratagene, La Jolla, CA, USA

2.1.6. Materials for cell culture

CD14+ immunomagnetic microbeads	Miltenyi Biotec, Bergisch Gladbach, Germany
Dulbecco's Modified Eagle's medium (DMEM)	Gibco, Grand Island, NY, USA
Endothelial cell basal medium	PromoCell, Heidelberg, Germany
Fetal bovine serum (FBS)	Biochrom KG, Berlin, Germany
Ficoll-Paque	Amersham Biosciences, Freiburg, Germany
Gentamycin	PromoCell, Heidelberg, Germany
Ham's F12-nutrient mixture	Gibco, Grand Island, NY, USA
Hepes	Gibco, Gaithersburg, MD, USA
Non-essential amino acids	Gibco, Gaithersburg, MD, USA
Penicillin-Streptomycin	PAA, Linz, Austria
RPMI 1640	Gibco, Gaithersburg, MD, USA
Sodium-pyruvate	Gibco, Gaithersburg, MD, USA

Trypsin-EDTA (1X)(w/o Ca & Mg)	Gibco, Grand Island, NY, USA
Tissue culture dish 6 cm	Falcon, Heidelberg, Germany
Tissue culture flash	Falcon, Heidelberg, Germany
Tissue culture 6-well plate (surface area 9.6 cm^2)	Greiner, Frickenhausen, Germany

2.1.7. *E.coli* strains and cell lines

RapidTrans™ competent *E. coli*		Activ Motive, Rixensart, Belgium
RAW264.7	mouse monocytic cells	ATCC, Manassas, VA, USA
ECV304	human endothelial cells	ECCC, Braunschweig, Germany
LLC-PK1	porcine kidney epithelial cells	ATCC, Manassas, VA, USA
HeLa	human cervix epithelial cells	ATCC, Manassas, VA, USA

H35 (T7-18) hepatoma cells were from Dr. Heinz Baumann (Roswell Park Cancer Institut, Buffallo, NY, USA)

2.1.8. Plasmids

pGL3 basic	Promega, Madison, WI, USA
pNF-κB	Stratagene, La Jolla, CA, USA
pcDNA3.1	Invitrogen, Karlsruhe, Germany
Gal4 plasmid system (pFR-luc, pFA-CHOP, pFC2-dbd)	
PathDetect® CHOP *trans*-Reporting system (#219015)	Stratagene, La Jolla, CA, USA

The following plasmid was provided by Dr. Craig A. Hauser (The Burnham Institute, La Jolla, USA):

pAP-1

The following plasmid was provided by Dr. Jawed Alam (Ochsner Clinic Foundation, New Orleans, LA, USA):

p3xStRE

The following plasmids were provided by Dr. Richard Gaynor (MD Anderson Cancer Institute, Houston, TX, USA):

NIKdn, IKKβdn, IκBαdn

The following plasmids were provided by Dr. Jiahuai Han (Scripps Research Institute, La Jolla, CA, USA):

(pcDNA3) p38α, p38α(AF), p38β, p38β(AF), p38γ, p38γ(AF), p38δ, p38δ(AF)

The following plasmids were provided by Dr. Axel Kahn (Institute Cochin, Paris, France):

(pCR3) USF2, USF2dn (ΔbTDU1)

The following plasmids were provided by Dr. Thomas Kietzmann (Fachbereich Chemie/ Biochemie, Universität Kaiserslautern, Germany):

(pAlter) p300wt, p300mut

(pCMV5-myc) PDK1dn, (EGB2T-AAA) PKB2dn, (pCMV3-myr) PKBca

2.1.9. Antibodies

First antibodies :

HO-1	(# SPA-895)	Stressgen, Victoria, BC, Canada
COX-2	(# 210-726-1)	Axxora Deutschland, Grünberg, Germany
GAPDH	(# 5G4)	HyTest, Turku, Finland
ERK	(# 9102)	Cell Signaling, Beverly, MA, USA
Phospho ERK	(# 9101)	Cell Signaling, Beverly, MA, USA
JNK	(# 9252)	Cell Signaling, Beverly, MA, USA
Phospho JNK	(# 9251)	Cell Signaling, Beverly, MA, USA
p38	(# 9212)	Cell Signaling, Beverly, MA, USA
Phospho p38	(# 9211)	Cell Signaling, Beverly, MA, USA
PKB	(# 9272)	Cell Signaling, Beverly, MA, USA
Phospho PKB (Ser473)	(# 9271)	Cell Signaling, Beverly, MA, USA
Phospho PKB (Thr308)	(# 9275)	Cell Signaling, Beverly, MA, USA

Secondary antibodies - horseradish peroxidase conjugated:

Goat anti-rabbit IgG-HRP	(# R1364HRP)	DPC Biermann, Bad Nauheim, Germany
Goat anti-mouse IgG-HRP	(# SP1041HRP)	DPC Biermann, Bad Nauheim, Germany

2.1.10. Other materials

3 mm Whatman paper	Schleicher & Schuell, Dassel, Germany
Lumi-Light^{PLUS} western blotting substrate	Roche, Mannheim, Germany
Polyvinylidene fluoride membranes (PVDF)	Millipore, Bedford, MA, USA
X-ray films X-Omat AR	Kodak, Rochester, NY, USA

2.1.11. Instruments

Blotting-semidry Whatman	Biometra, Göttingen, Germany
Centrifuge : RC5C	Sorvall®, Wiesloch, Germany
Hettich Rotixa/RP	Hettich, Tuttlingen, Germany
Hettich Mikro 22R	Hettich, Tuttlingen, Germany
Electrophoresis apparatus	Bio-Rad, Munich, Germany
Incubator CO_2	Heraeus, Hanau, Germany
Laminair® HB2448	Heraeus, Hanau, Germany
Luminometer, Lumat LB 9507	Berthold Technologies, Bad Wildbad, Germany
Microscope Axiovert 10	Zeiss, Oberkochen, Germany
Spectrophotometer	Beckmann, Munich, Germany

2.2. Methods

2.2.1. Transformation and preparation of plasmid DNA

2.2.1.1 . Medium and solutions

Luria Broth (LB)-Medium:
1 % (w/v) Bacto-tryptone
1 % (w/v) NaCl
0.5 % (w/v) Yeast extract
pH 7.5, autoclaved

LB-Agar:
1 % (w/v) Agar in LB medium, autoclaved
100 μg/ml Ampicillin

Solution 1: 50 mM glucose
25 mM Tris-Cl, pH 8
10 mM EDTA
fresh 0.33 μg/μl RNAse A

Solution 2 : 1% SDS in 0.2 N NaOH

Solution 3: 3 M potassium acetate
11.5% glacial acetic acid

2.2.1.2. Transformation of competent *E. coli*

The transformation reaction tube was removed from -80˚C storage and placed on ice to thaw. 1-5 μg of plasmid DNA was added to thawed cells, mixed by tapping the tube gently and was placed on ice immediately. The transformation reaction was incubated on ice for 30 min, the tube was heat-shocked by immersing in a 42˚C water bath for exactly 30 s and then the tube was placed on ice for 2 min. 250 μl LB medium was added and the tube was incubated at 37˚C for 1 h with shaking at 225-250 rpm. Using a sterile spreader, 20-200 μl of transformation was plated out on a pre-warmed LB agar plate and the plate was allowed to completely absorb any excess media. Inverted plates were incubated overnight at 37˚C.

2.2.1.3. Mini preparation of plasmid DNA

A single bacterial colony was inoculated into 3 ml of selective LB-medium and grown with vigorous shaking for 16 to 18 h. 700 μl culture bacteria was mixed with 300 μl 50% glycerol and stored in -80°C as frozen stock plasmid DNA. 1 ml culture bacteria was centrifuged at 14,000 rpm for 30 s. The pellet was resuspended in 150 μl solution 1, incubated for 5 min at room temperature (RT), 150 μl solution 2 was added, incubated for 5

min at RT, 150 μl solution 3 was added, incubated for 5 min at RT, and then centrifuged for 5 min. 1 ml cold ethanol (100%) was added to the supernatant, vortexed and incubated for 10 min at RT and centrifuged for 10 min. The pellet was washed with 1 ml ethanol (70%) and centrifuged at maximum speed for 2 min, the supernatant was removed and air-dried pellet for 10 min. 20 to 30 μl of 10 mM Tris-Cl (pH 8.0) was added to the pellet and incubated for 2 min at RT.

Plasmids preparations were performed with QIAGEN Plasmid Midi or Maxi kits. The preparation procedure was according to the manufacturer's protocol. The concentration of DNA was calculated by the measurement of the absorbance at 260 nm and 280 nm.

2.2.1.4. Agarose gel electrophoresis

6x loading buffer:

0.025% (w/v) bromophenol blue
30% (v/v) glycerol

50x TAE:

242 g Tris base
57.1 ml glacial acetic acid
100 ml 0.5 M EDTA (pH 8.0)

Agarose gels (0.8-2%) were used for analyzing plasmid DNA. The agarose was melted in 1x TAE buffer using a microwave. Before the gel was poured into a casting platform, ethidium bromide was added at a dilution of 1:10,000. DNA samples were mixed with an appropriate amount of 6x loading buffer before loading into the wells. The voltage of electrophoresis was set to 10 V/cm. DNA was visualized by placing the gel on a UV light source and photographed.

2.2.2. Preparation of luciferase promoter constructs

Construction of HO-1 promoter plasmids

pHO-1338:

The construct of the rat HO-1 promoter 5'-flanking region from -1338 to +71 was amplified by PCR from rat genomic DNA by using the oligonucleotide 5'-CTCAGGATTAACAAAACAAAGACACAAAAAG-3' (-1338/-1309) as forward and 5'-GAGATGGCTCTGCTCCGGCAGGCTCCACTC-3' (+42/+71) as reverse primer. The resulting PCR product was blunted by Klenow enzyme and was phosphorylated with T4 polynucleotide kinase. This DNA-fragment was ligated into the *Sma*I site of pUC18. The insert was excised with *Kpn*I/*Bam*HI and cloned into the *Kpn*I/*Bgl*II site of pGL3basic (Immenschuh et al., 2000).

pHO-754:

The rat HO-1 promoter 5'-flanking region from -754 to +71 was amplified by PCR from rat genomic DNA by using the oligonucleotide 5'-GCCAGGAATTCGGAGGGTAATTGTCCAAG-3' (-754/-726) as forward and 5'-GAGATGGCTCTGCTCCGGCAGGCTCCACTC-3' (+42/+71) as reverse primer. The resulting PCR product was blunted by Klenow enzyme and phosphorylated with T4 polynucleotide kinase. This DNA-fragment was ligated into the *Sma*I site of pUC18. The insert was excised with *Kpn*I/*Bam*HI and cloned into the *Kpn*I/*Bgl*II site of pGL3basic (Immenschuh et al., 1998).

pHO-754Am:

This plasmid was constructed by using PCR-based mutagenesis. The forward primer 5'-**t**AG**a**CTCCGGTACTCAGGCA-3' (-658/-639), containing a C to T and a T to A conversion at position 1 and 4 respectively, and the reverse primer 5'-CACATGGCTCTGACACATCTATAAC-3', containing the wild type HO-1 sequences -668/-692, were used with the ExSite Mutagenesis kit (Immenschuh et al., 1998).

pHO-754Em:

The luciferase construct pHO-754Em was generated with the QuickChange™ XL Site-Directed Mutagenesis Kit using the oligonucleotide 5'-GGCTCAGCTGGGCGGCCAC**ctctag**ACTCGAGTAC-3' (Kietzmann et al.,2003).

pHO-754Am/Em:

The luciferase reporter gene construct pHO-754Am/Em was generated from pHO-754Am with the QuickChange™ XL Site-Directed Mutagenesis Kit using the oligonucleotide 5'-GGCTCAGCTGGGCGGCCAC**ctctag**ACTCGAGTAC-3'.

pHO-347:

The luciferase reporter gene construct pHO-347 was generated by deletion of the -754/-347 fragment of HO-754 by *Apa*I and *Kpn*I followed by blunting of the remaining vector with Klenow enzyme and phosphorylation with T4 polynucleotide kinase. This DNA fragment was ligated into the *Sma*I site of pUC18. The insert was excised with *Kpn*I/*Bam*HI and cloned into the *Kpn*I/*Bgl*II site of pGL3basic (Kietzmann et al., 2003).

pHO-50:

This plasmid was generated by PCR using the representative primers.

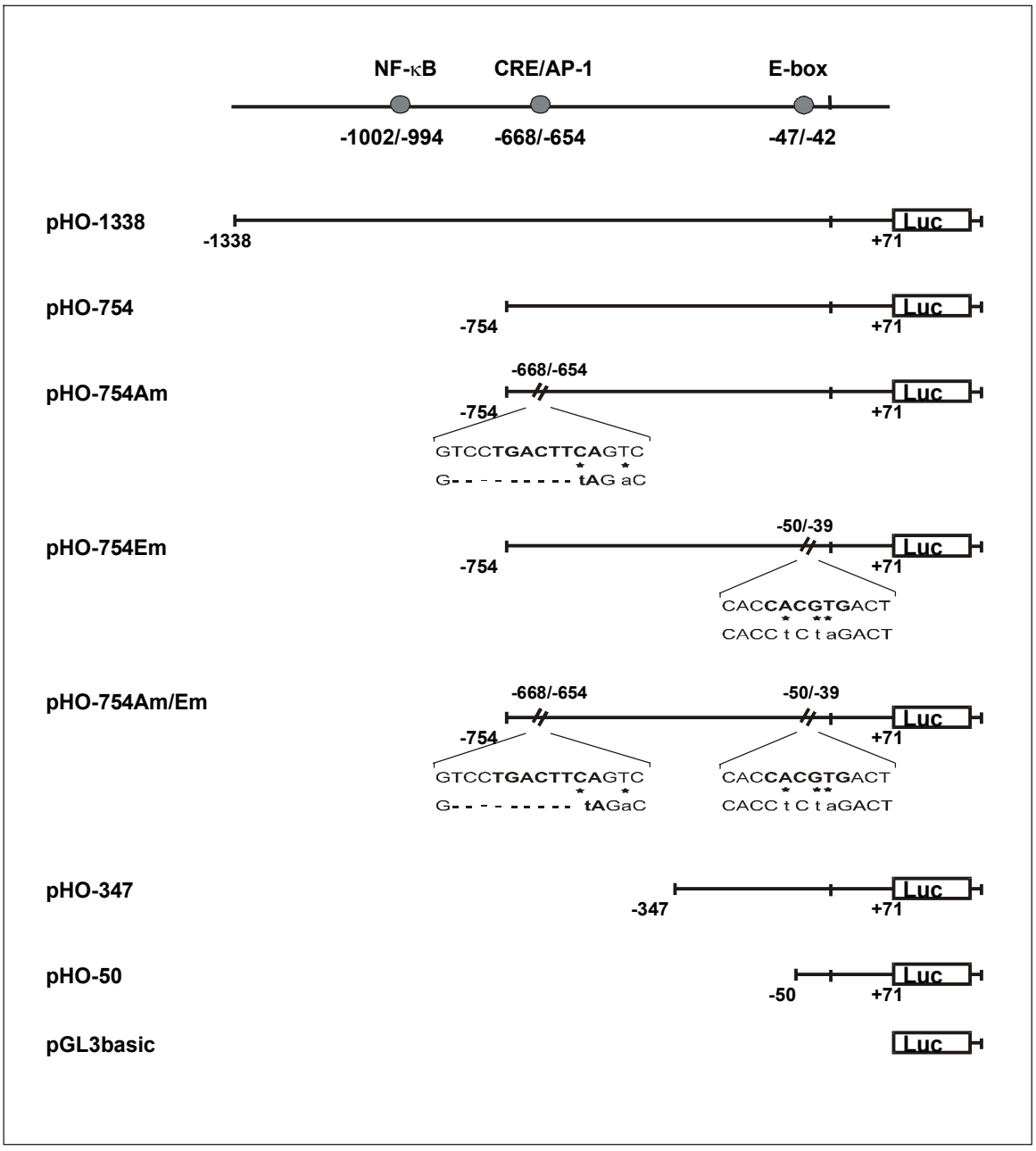

Fig. 4. HO-1 promoter luciferase gene construct. The wild type HO-1 sequence is shown on the upper strand, deleted bases are indicated by – and mutated bases are shown in *lowercase letters* and indicated by *.

2.2.3. Generation of human peripheral blood monocytes (PBMC) and cell culture of cell lines

Human PBMC were isolated from buffy coats of healthy blood donors by Ficoll-Paque density gradient centrifugation as described previously (Steinschulte et al., 2003). CD14+ monocytes were purified (>95%) using CD14+ immunomagnetic microbeads and 3 x 10^6 cells were cultured in 6-well flat-bottom plates with 3 ml RPMI 1640 with L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, sodium-pyruvate, non-essential amino acids and 10% FBS. After 2 days, 50% supernatant was replaced with medium containing either AEBSF (250 µM), LPS (1 µg/ml) or TPA (0.5 µM).

RAW264.7 cells were grown in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. Cell cultures were kept under air/CO2 (19:1) at 100% humidity. Treatment of cells with LPS or other reagents was performed with serum-free medium at the indicated concentrations.

HeLa and H35 (T7-18) cells were grown in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. LLC-PK1 cells were cultured in Ham's F12 with 20% DMEM, 15% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. ECV304 cells were cultured in endothelial cell basal medium with 5% FBS and 1% Gentamycin (5000 µg/ml).

2.2.4. Western blotting

2.2.4.1. Protein preparations from cell cultures

After washing cell cultures twice with 0.9% NaCl, 300 µl per-well of lysis buffer (0.05 M Tris-Cl pH 6.8, 2% SDS, 10% glycerol, bromophenolblue, 0.2 M DTT, add fresh 4% protease inhibitors) was added and cells were scraped from culture dishes and then homogenized by passing through a 25-gauge needle. The homogenate was incubated for 3 min at 95°C and the protein concentration was determined in the supernatant by the Bradford method.

2.2.4.2. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

2.2.4.2.1. Solutions

1.5 M Tris-Cl pH 8.8 and pH 6.8 (100 ml):
18.15 g Tris base
50 ml H_2O
adjusted pH to 8.8 or 6.8 with HCl, filled to 100 ml with H_2O

10 x SDS running buffer (1 l)
10 g SDS
30.3 g Tris base
144.1 g glycine

dissolved in 5 l H_2O

Resolving gels (mini gel):

	8%	**12%**
H_2O	2.3 ml	1.6 ml
30% acrylamide	1.3 ml	2.0 ml
1.5 M Tris-Cl pH 8.8	1.3 ml	1.3 ml
10% SDS	0.05 ml	0.05 ml
10% APS	0.05 ml	0.05 ml
TEMED	0.003 ml	0.002 ml

Stacking gels 5% (mini gel):

H_2O	1.4 ml
30% Acrylamide	0.33 ml
1.5 M Tris-Cl pH 6.8	0.25 ml
10% SDS	0.02 ml
10% APS	0.02 ml
TEMED	0.002 ml

2.2.4.2.2. Resolving and stacking gel

Immediately after addition of TEMED and APS, the running gel solution was poured between the glass plates and overlayed carefully with ethanol (70%). After polymerization ethanol was removed from the surface of the polymerized gel with a sheet of filter paper. The stacking gel was poured on top of the resolving gel and the comb was inserted between the glass plates. The electrophoresis apparatus was assembled according to manufacturer's instructions. Total protein (30-60 µg) was loaded onto SDS-polyacrylamide gel (10-12%). The voltage of electrophoresis was set to 15 – 25 mA for the stacking gel and to 50 – 60 mA for the resolving (separation) gel.

2.2.4.3. Semi-dry transfer blotting

Transfer buffer:

5.8 g Tris base
2.9 g glycine
0.37 g SDS
200 ml methanol
dissolved in 1 l H_2O

The gel was immersed in transfer buffer and equilibrated for 15 min. The immobilon-P membrane, PVDF was immersed in 100% methanol for 15 s and transferred to a container of H_2O for 2 min, then the membrane was equilibrated for at least 5 min in transfer buffer. Three sheets of Whatman paper were immersed in transfer buffer and placed in the center of the graphite anode electrode plate. The PVDF membrane was placed on top of the Whatman paper and the gel was placed on top of the membrane. Three pieces of Whatman paper were soaked in the transfer buffer and placed on top of the gel. The cathode plate cover was

placed on top of the assembled transfer stack. The current was set at 0.8 mA/cm^2 for 1 to 2 h.

2.2.4.4. Visualizing the protein

The blot was stained with Ponceau S to assess the quality of the transfer. The blot was stained in a solution of 0.5% Ponceau S, 1% acetic acid for 1 min and destained in H_2O to the desired contrast or washed with 0.1 N NaOH to remove the stain completely.

2.2.4.5. Immunodetection

10x Tris buffer saline (TBS):
24.2 g Tris base
80 g NaCl
adjusted to pH 7.6 with HCl, filled to 1 l with H_2O.

1x Tween-TBS (TTBS):
100 ml 10x TBS
900 ml H2O
0.5 - 1 ml Tween 20

The primary antibodies were used in the following dilutions:

HO-1	1:1,000
COX-2	1:1,000
GAPDH	1:1,000
ERK	1:500
Phospho ERK	1:500
JNK	1:500
Phospho JNK	1:500
p38	1:500
Phospho p38	1:500
PKB	1:500
Phospho PKB (Ser473)	1:500
Phospho PKB (Thr308)	1:500

Secondary antibodies - horseradish peroxidase conjugated were used in the following dilution:

Goat anti-rabbit IgG	1:20,000-100,000
Goat anti-mouse IgG	1:20,000

The PVDF membrane was soaked by a short rinse in methanol, isopropanol or ethanol and then washed with H_2O. Membranes were blocked with 1x TTBS containing 5% dry milk for 30 min at RT under constant shaking. The primary antibodies were applied in 5% dry milk or 5 % BSA for 1 h at RT or overnight in 4^{o}C. Then, the membrane was washed in 1x TTBS for 3 x 2 min and incubated with secondary antibodies for 1 h at RT after which membranes were washed in 1x TTBS for 3 x 2 min.

The membrane was placed between 2 sheets of transparency film and Lumi-Light^{plus} substrate was added onto the membrane, the transparency film was sealed and incubated

for 5 to 30 min. Excess liquid was squeezed out and the membrane was exposed on X-ray films for 5 s up to 1 h.

2.2.4.6. Stripping and reusing membranes

The membrane was washed for 5 min in H_2O, transferred to 0.2 M NaOH for 5 min and then washed again in H_2O for 5 min. The membrane was then ready for another immunoprobing procedure. Non-fat dry milk was used as blocking reagent to effectively reprobe the membranes.

2.2.5. Transfection and luciferase assay

2.2.5.1. Transfection

Cells were plated in 6-well plates 24 h before the transfection experiment. Transfection of plasmid DNA into RAW264.7 cells was performed by the liposome method using FuGENE 6 transfection reagent. DMEM serum-free medium was added to a total volume of 110 μl (for one well) in an eppendorf tube and 0.5 to 1 μg of reporter plasmid DNA was added. The contents was mixed by pipetting several times. One to 2 μl of FuGENE 6 was added directly into this mixture, mixed by pipetting and incubated for another 15 min at RT. Prior to use, FuGENE 6 reagent was mixed gently by inversion. The DNA-lipid complex was added in drops to the cells in the presence of medium with FBS (100 μl volume per-well), distributed around the well and swirled to ensure even dispersal. After transfection cell culture was continued for 24 h before cell harvest or treated with various stimuli, as indicated.

For cotransfection experiments 0.1 to 1.5 μg plasmid DNA of the indicated expression vectors was added. The amount of transfection reagent in proportion to the amount of total μg DNA was increased accordingly.

2.2.5.2. Luciferase activity assay

2.2.5.2.1. Preparation of cell lysates

Luciferase cell culture lysis reagent (CCLR) was supplied as a concentrate (5x). The working concentration was prepared by adding 1 volume of CCLR 5x to 4 volumes of H_2O, equilibrated lysis reagent to RT before use. The growth medium from transfected culture cells was removed carefully and cells were rinsed with 0.9 % NaCl. Sufficient CCLR was added (200-300 μl per-well of a 6-well plate) to cover the cells. The 6-well plate was rocked several times to ensure complete coverage of the cells with lysis buffer. Cells were scrapped from the culture dishes and transferred to a microcentrifuge tube. The tube was placed on ice and centrifuged at 12,000x g for 15 s at RT or for up to 2 min at 4°C. The cell lysates were assayed directly.

2.2.5.2.2. Luciferase assay

Luciferase assay substrate was reconstituted with 10 ml luciferase assay buffer. This luciferase assay reagent was mixed well and equilibrated at RT before use. The luminometer injector was primed with luciferase assay reagent and 10 to 50 µl of cell lysate was dispensed into a luminometer tube. The tube was placed in the luminometer and reading was initiated by injecting 50 to 100 µl of luciferase assay reagent into the tube. The luminometer was programmed to perform a 2 s measurement delay followed by a 10 s measurement read for luciferase enzyme activity. The reading time was adjusted to work in a linear range in the respective experiment. Relative light units of luciferase activity were normalized with sample protein.

2.2.6. Determination of protein concentration

Sample protein was pre-treated with Compat-Able™ protein assay preparation reagent kit to remove interfere substance and the protein assay was performed with DC protein assay reagent kit, this assay is based on the Lowry method. The preparation was performed according to the manufacturer's instructions and concentration of protein was calculated by measurement of the absorbance at the wavelength 750 nm.

2.3. Statistical analysis

Autoradiographic signals from Western blots were evaluated by videodensitometry scanning and quantitation with Imagequant® software. The relative densities of bands were expressed as fold-induction normalized to GAPDH from at least three independent experiments. Data were analysed by Student's *t* test for paired values and presented as means ± S.E. A value of $p \leq 0.05$ was considered as statistically significant.

Data obtained from luciferase activity measurement were analyzed by Student's *t* test for paired values and presented as means ± S.E. from at least three independent experiments with duplicates of each point. A value of $p \leq 0.05$ was considered as statistically significant.

3. RESULTS

3.1. LPS-dependent induction of HO-1 gene expression in RAW264.7 cells

It has previously been demonstrated that HO-1 gene expression is induced by LPS in mononuclear phagocytes (Camhi et al., 1995; 1998). To investigate the regulatory mechanisms of LPS-dependent HO-1 gene induction in more detail, the monocytic cell line RAW264.7 was applied for the experiments of the present study. Treatment with LPS induced HO-1 protein expression in RAW264.7 cells as determined by Western blot analysis (Fig. 5A). Up-regulation of HO-1 protein levels was time-dependent with a maximum at 18 h (Fig. 5B). HO-1 gene expression was also induced by treatment with LPS on the mRNA level (data not shown).

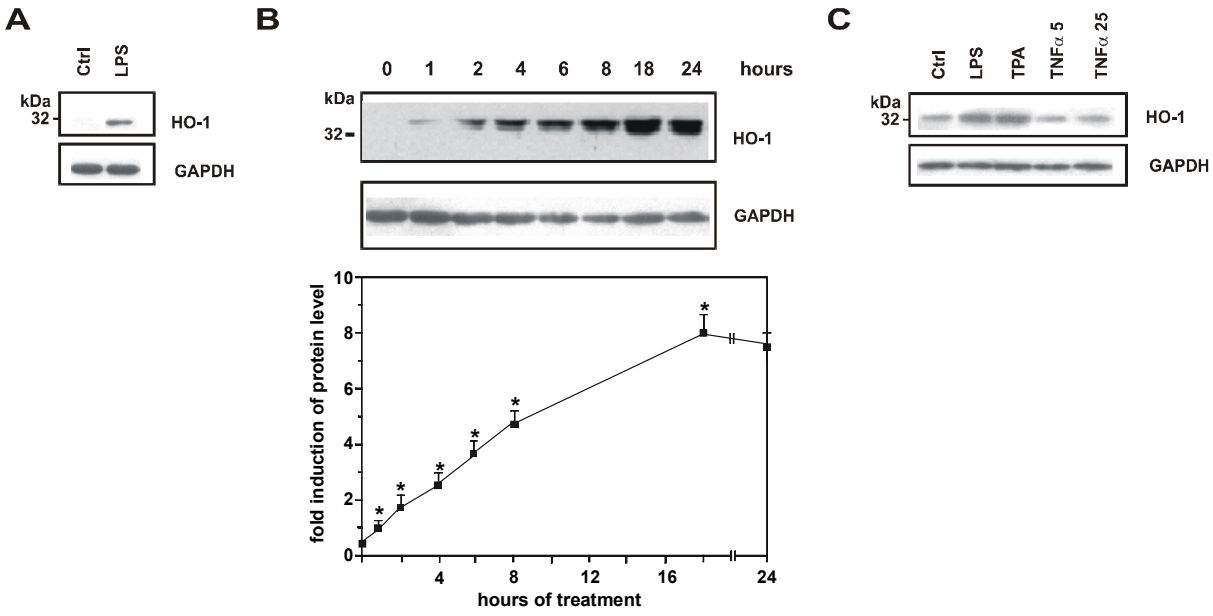

Fig. 5. LPS-dependent induction of endogenous HO-1 gene expression in RAW264.7 cells. (A) RAW264.7 cells were cultured as described in Materials and Methods. Cells were treated with LPS (1 μg/ml) or control medium for 18 h, as indicated or, (B) in the presence of LPS (1 μg/ml) for the times indicated, or (C) with LPS (1 μg/ml), TPA (0.5 μM), TNFα (5 μg/ml and 25 μg/ml) or control medium, as indicated. Total protein (60 μg) was subjected to Western blot analysis and probed sequentially with antibodies against rat HO-1 and GAPDH. *, significant differences LPS *versus* control. *Ctrl*, control.

Since LPS-dependent induction of HO-1 gene expression may be mediated via an autocrine mechanism by the proinflammatory cytokine TNFα, the effect of LPS was compared with that of TNFα. As demonstrated in Fig. 5C, treatment with TNFα had no effect on HO-1 gene expression. As a control the phorbol ester TPA, which is a known inducer of HO-1 gene expression (Alam and Den, 1992), strongly up-regulated HO-1 protein expression (Fig. 5C). LPS-dependent induction of HO-1 gene expression appeared to be cell-specific, because no induction was observed in cell cultures of LLC-PK1 (porcine kidney epithelial cells), H35 (T7-18) (hepatoma cells), HeLa (human cervix epithelial cells) and human endothelial cells (ECV304) (data not shown). The data suggest that LPS induces the endogenous HO-1 gene expression in RAW264.7 cells.

3.2. Transcriptional induction of HO-1 gene expression by LPS

Up-regulation of HO-1 gene expression by most stimuli occurs on the transcriptional level (Choi and Alam, 1996; Sikorski et al., 2004). Accordingly, pretreatment of RAW264.7 cells with the transcription inhibitor actinomycin D prevented the LPS-dependent HO-1 mRNA induction (data not shown). To further investigate the molecular mechanisms of LPS-dependent HO-1 gene induction, the effect of LPS was determined in transiently transfected RAW264.7 cells with luciferase reporter gene constructs with the proximal 1338 bp of the rat HO-1 gene promoter (pHO-1338). For a comparison, RAW264.7 cells were transfected with reporter gene constructs containing four copies of the NF-κB consensus sequences (pNF-κB), three copies of the HO-1 antioxidant RE (ARE) (p3xStRE) or three copies of the AP-1 consensus sequence (pAP-1) (Fig. 6). A marked LPS-dependent induction of luciferase activity was determined for pHO-1338 and a lower level of induction was observed for pAP-1, pNF-κB and p3xStRE. The data suggest that activation of HO-1 gene expression by LPS in RAW264.7 cells is transcriptionally regulated via regulatory DNA sequences of the proximal HO-1 promoter region.

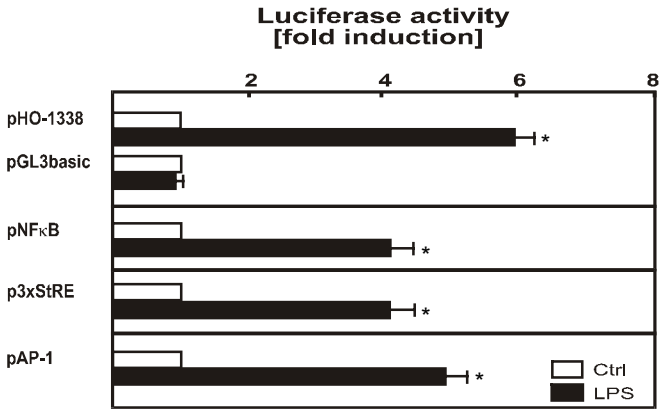

Fig. 6. LPS-dependent induction of rat HO-1 promoter activity. RAW264.7 cells were transiently transfected with reporter gene constructs containing the proximal 1338 bp of the rat HO-1 gene promoter region (pHO-1338), four copies of the consensus sequence of NF-κB (pNF-κB), three copies of the HO-1 stress RE (p3xStRE), three copies of the AP-1 consensus motif (pAP-1) or empty control vector pGL3basic. 24 h after transfection, cells were treated for 18 h with or without LPS (1 μg/ml), as indicated. Cell extracts were assayed for luciferase activity and the -fold induction in each experiment relative to the control was determined. *, significant differences treatment *versus* control. *Ctrl*, control.

3.3. Regulatory role of the NF-κB signaling pathway for LPS-dependent activation of HO-1 promoter activity

The NF-κB signaling pathway has previously been shown to play an important regulatory role for LPS-dependent gene regulation (Karin and Ben-Neriah, 2000). Therefore, the effect of the pharmacological NF-κB inhibitors caffeic acid phenethyl ester (CAPE) and Nap-tosyl-L-lysine chloro-methylketone (TLCK) on LPS-dependent induction of HO-1 promoter activity was determined in RAW264.7 cells. Up-regulation of HO-1 promoter activity by LPS was inhibited by pretreatment with CAPE and TLCK in a dose-dependent manner (Fig. 7). For comparison, the regulatory pattern of the reporter gene construct pNF-κB by these inhibitors is shown (Fig. 7A and B, right panel).

To further examine the regulatory role of the NF-κB signaling pathway for LPS-dependent HO-1 induction, cotransfection experiments were performed with expression vectors for dominant negative forms of upstream kinases of the NF-κB pathway, NIK, IKKβ and IκBα. As demonstrated in Fig. 8, LPS-dependent induction of HO-1 promoter activity was markedly decreased by overexpressed dominant negative NIK, IKKβ and IκBα. Taken together, the data demonstrate that activation of the NF-κB signaling pathway plays an important role for LPS-dependent induction of HO-1 promoter activity.

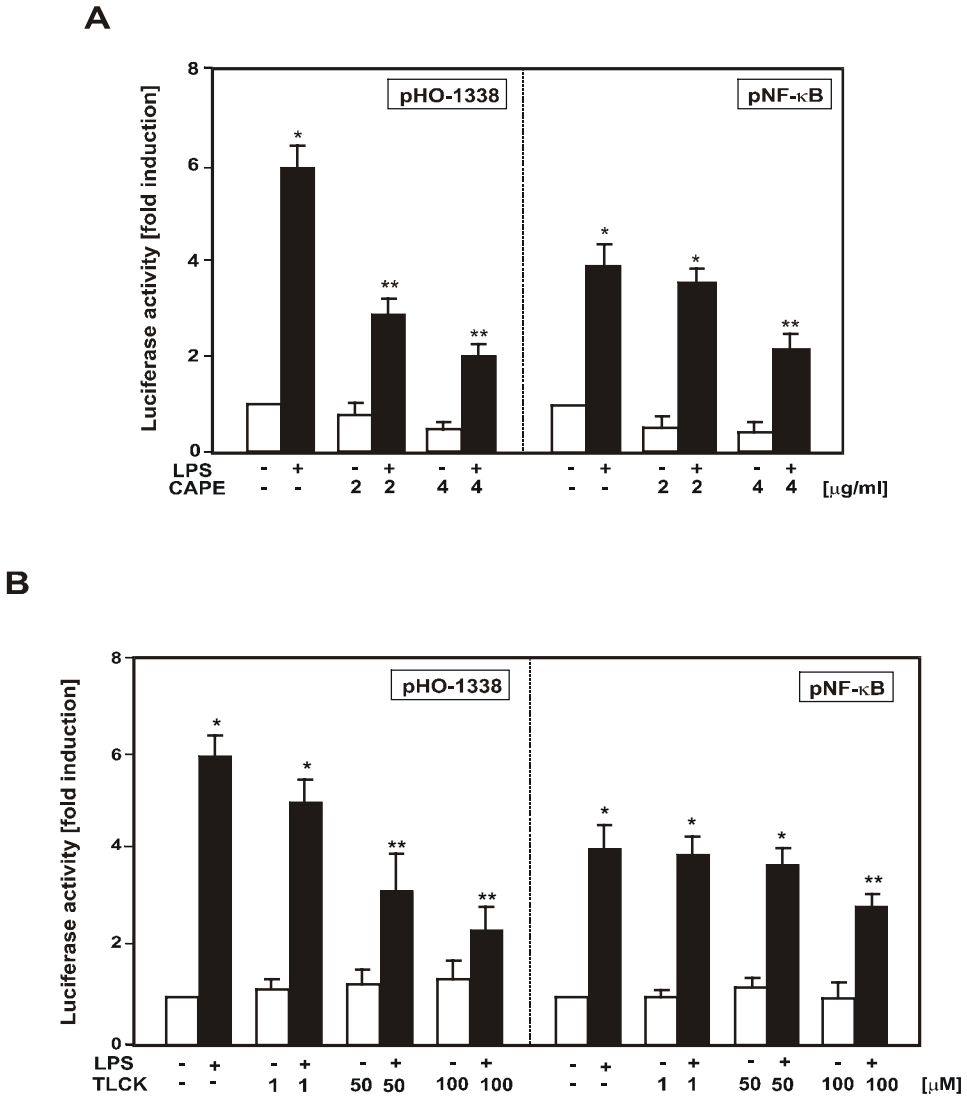

Fig. 7. Effect of pharmacological NF-κB inhibitors on LPS-dependent induction of HO-1 promoter activity in RAW264.7 cells. RAW264.7 cells were transiently transfected with the HO-1 reporter gene construct pHO-1338 or pNF-κB. 24 h after transfection cells were pretreated with the NF-κB inhibitors (A) CAPE and (B) TLCK at the indicated concentrations for 1 h. Then, cells were incubated for another 18 h in the absence or presence of LPS (1 μg/ml). Cell extracts were assayed for luciferase activity and the -fold induction relative to the control was determined. *, significant differences treatment *versus* control; **, TLCK + LPS *versus* LPS.

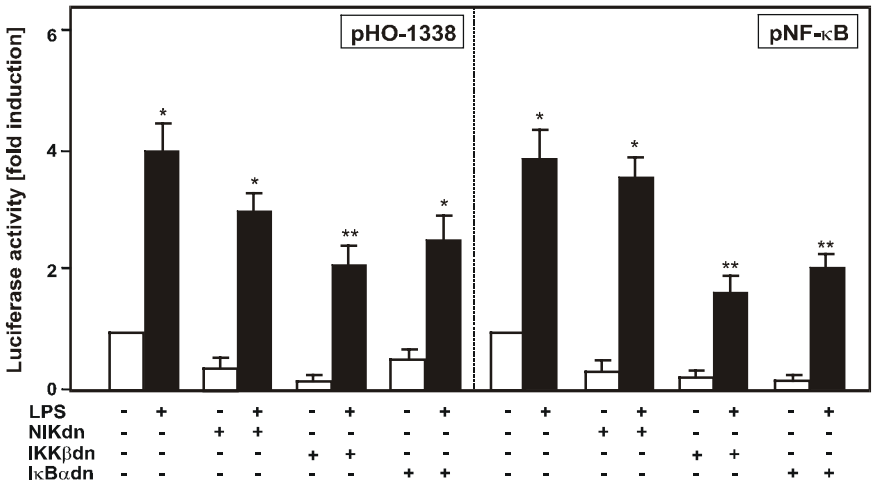

Fig. 8. Inhibition of HO-1 promoter activity by overexpressed dominant negative mutants of NIK, IKKβ and IκBα. RAW264.7 cells were cotransfected with the reporter gene constructs pHO-1338 or pNF-κB and expression vectors with dominant negative mutants of NIK, IKKβ and IκBα, as indicated. 24 h after transfection cells were treated with or without LPS (1 μg/ml) for another 18 h. Cell extracts were assayed for luciferase activity and the -fold induction relative to the control was determined. *, significant differences treatment *versus* control; **, LPS + IKKβdn *versus* LPS control , LPS + IκBαdn *versus* LPS control. *NIKdn*, dominant negative NIK; *IKKβdn*, dominant negative IKKβ; *IκBαdn*, dominant negative IκBα.

3.4. Activation of MAPKs by LPS in RAW264.7 cells

To determine the potential role of MAPKs for LPS-dependent induction HO-1 gene expression, RAW264.7 cells were treated with LPS for various lengths of time and cell extracts were analyzed for phosphorylated and total MAPKs. An increase in the level of phosphorylated ERK1 and ERK2 was observed in LPS-treated cells reaching a maximum after 15 min (Fig. 9). Moreover, phosphorylation of JNK and p38 was observed in LPS-treated cells (Fig. 9), suggesting that the three major MAPK pathways are activated by LPS in RAW264.7 cells.

To investigate the regulatory role of MAPKs for LPS-dependent regulation of HO-1 gene expression, various pharmacological MAPK inhibitors were applied. Pretreatment of cells for 1 h with the p38 MAPK inhibitor SB203580 strongly reduced LPS-dependent induction of HO-1 promoter activity. By contrast, pretreatment with the MEK inhibitor PD98059 and the JNK inhibitor SP600125 did not affect LPS-dependent induction of HO-1

promoter activity (Fig. 10A). The data suggest that the p38 signaling pathway plays a major regulatory role for the induction of HO-1 by LPS.

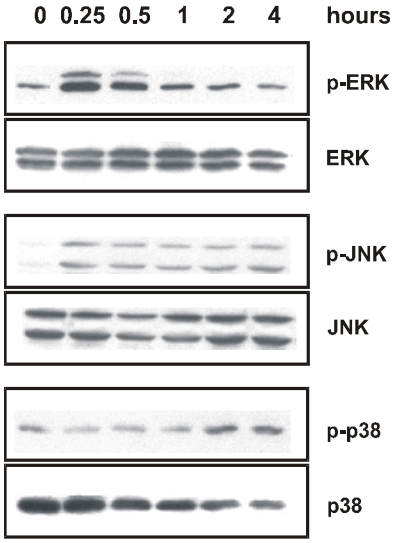

Fig. 9. Activation of MAPKs by LPS in RAW264.7 cells. RAW264.7 cells were cultured as described in Materials and Methods and were treated with LPS (1 μg/ml) for the times indicated. Total protein (60 μg) was subjected to Western blot analysis and probed with antibodies for various MAPKs. Membranes were initially used to detect phosphorylated MAPKs, then stripped, and probed with antibodies against total MAPKs. Autoradiograms of a representative experiment are shown. *p-ERK*, phospho ERK; *p-JNK*, phospho JNK; *p-p38*, phospho p38.

3.5. Opposite regulatory roles of p38β and p38δ on LPS-dependent induction of HO-1 gene expression

To further elucidate the regulatory role of p38 for LPS-dependent HO-1 gene induction, the effect of overexpressed dominant negative mutants (AF) of the p38α, β, γ, and δ isoforms was examined. As demonstrated in Fig 9B, dominant negative p38β inhibited, whereas dominant negative p38δ increased, LPS-dependent induction of HO-1 promoter activity (Fig. 10B). These effects of dominant negative p38β and p38 were also confirmed for the endogenous HO-1 gene expression in RAW264.7 cells (Fig. 10C), indicating that p38β and p38δ have opposite regulatory roles for the LPS-dependent induction of HO-1 gene expression.

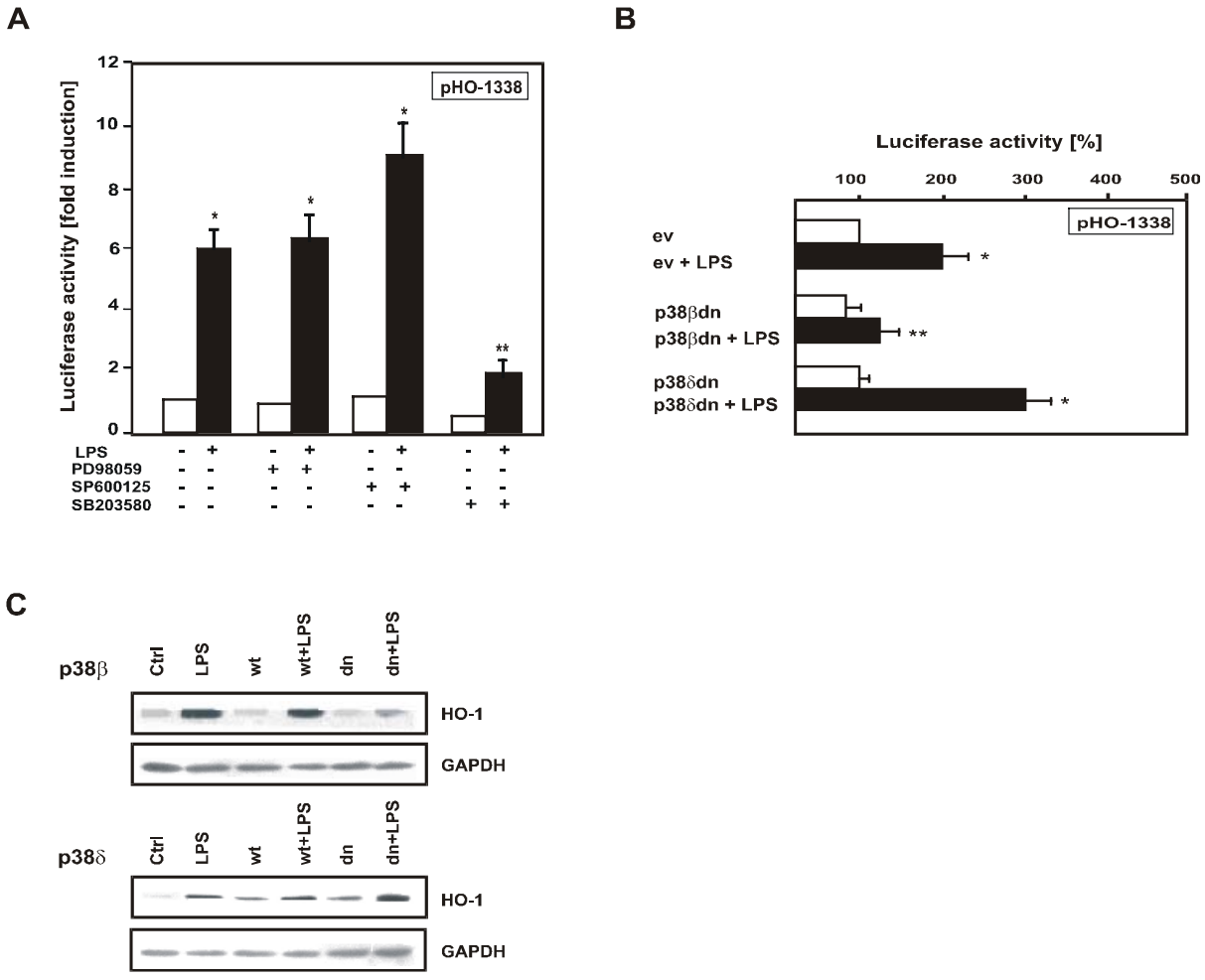

Fig. 10. Effect of MAPKs inhibitors on HO-1 promoter activity and role of p38β MAPK in the regulation of HO-1 promoter activity by LPS. (A) RAW264.7 cells were transiently transfected with the HO-1 reporter gene construct pHO-1338. 24 h after transfection cells were pretreated with the MEK inhibitor PD98059 (20 μM), JNK inhibitor SP600125 (10 μM) and p38 MAPK inhibitor SB203580 (20 μM) for 1 h. Then, cells were incubated for another 18 h in the absence or presence of LPS (1 μg/ml), as indicated. Cell extracts were assayed for luciferase activity and the -fold induction relative to the control was determined. *, significant differences LPS *versus* control; **, SB203580 + LPS *versus* control. (B) RAW264.7 cells were cotransfected with pHO-1338 and expression vectors for dominant negative p38β, p38δ or empty expression vector, as indicated. 24 h after transfection cells were treated with or without LPS (1 μg/ml) for another 18 h. Cell extracts were assayed for luciferase activity and the -fold induction relative to the control was determined. *, significant differences treatment *versus* control; **, LPS + p38βdn *versus* LPS control. (C) Endogenous HO-1 protein levels were detected in RAW264.7 that were transfected with expression vectors of wild-type and dominant negative of p38β or p38δ. 24 h after transfection cells were treated in the absence or presence of LPS (1 μg/ml), as indicated. Total protein (60 μg) was subjected to Western blot analysis with antibodies against rat HO-1 or GAPDH. Autoradiograms of a representative experiment are shown, respectively. *Ctrl*, control; *dn*, dominant negative; *ev*, empty vector; *p38βdn*, dominant negative p38β; *p38δdn*, dominant negative p38δ; *wt*, wild-type.

3.6. Role of the NF-κB, CRE/AP-1 and E-box elements for LPS-dependent induction of HO-1 promoter activity

To identify potential regulatory DNA sequences that may mediate the LPS-dependent induction of HO-1 gene expression, reporter gene constructs with serial deletions of the proximal HO-1 promoter 5'-flanking region were transiently transfected into RAW264.7 cells. Deletion of a putative NF-κB site (-1002/-994) did not alter the LPS-dependent induction of luciferase reporter gene activity. The responsiveness of HO-1 reporter gene constructs to LPS, however, was markedly reduced after deletion of DNA sequences from -754 to -347 and from -347 to -50. Remarkably, even a construct with 50 bp of the proximal HO-1 promoter gene region which contained an E-box motif (-47/-42) was slightly up-regulated by LPS (Fig. 11A). The TF upstream stimulatory factor (USF) has previously been shown to mediate LPS-dependent gene regulation (Potter et al., 1991; Goldring et al., 2000; Potter et al., 2003) and that of a previously identified HO-1 CRE/AP-1 site (-668/-654) (Immenschuh et al., 2000; Kietzmann et al., 2003).

To further assess the functional roles of an HO-1 E-box motif (-47/-42) and the CRE/AP-1 site (-668/-654), the LPS-dependent regulation of HO-1 reporter gene constructs with targeted mutations of these sites was examined in more detail. As demonstrated in Fig. 11B, LPS-dependent induction of luciferase activity of pHO-754Em was not reduced as compared to wild-type pHO-754. By contrast, induction of construct pHO-754Am/Em in response to LPS was markedly decreased. The data suggest that activation of HO-1 gene expression by LPS in RAW264.7 cells is mediated via regulatory DNA sequences of the proximal HO-1 promoter region.

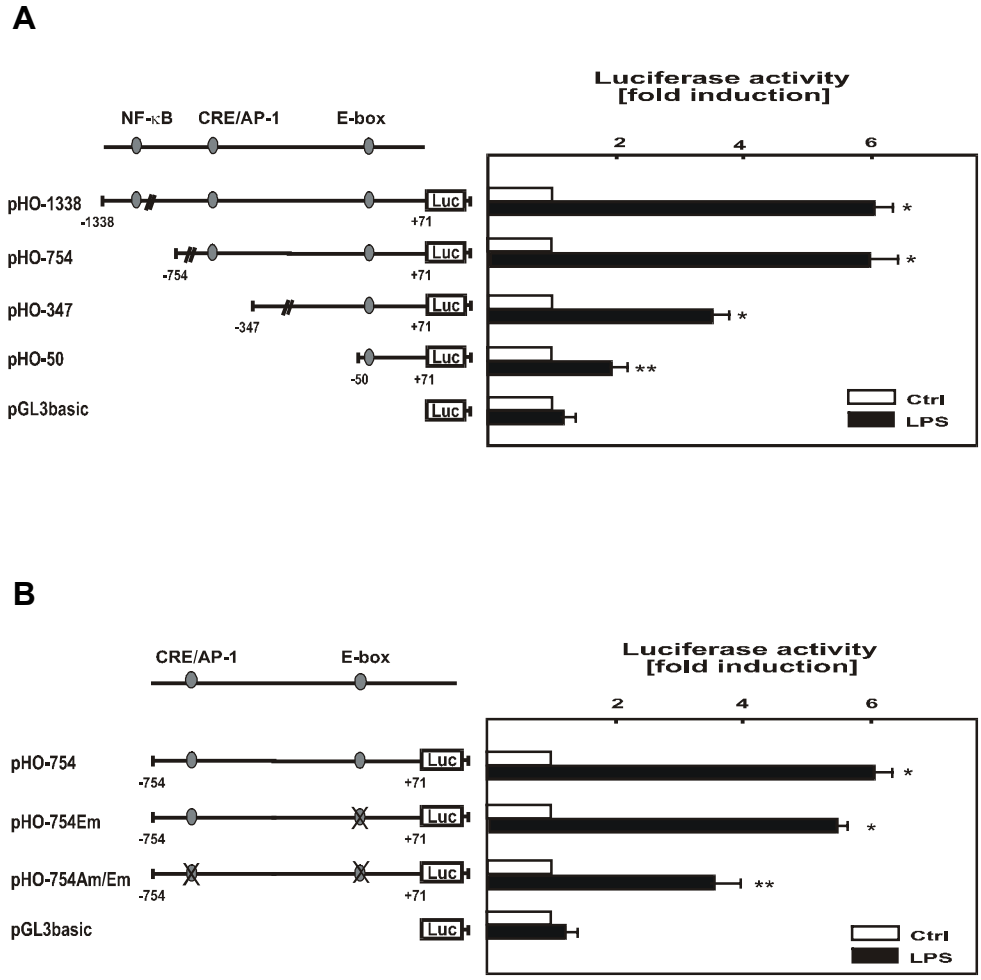

Fig. 11. Regulation of rat HO-1 gene promoter sequences by LPS in transiently transfected RAW264.7 cells. (A) The indicated serially deleted rat HO-1 reporter gene constructs were transiently transfected into RAW264.7 cells. 24 h after transfection cells were treated for 18 h with or without LPS (1 μg/ml). Localizations of a putative NF-κB sequence, the CRE/AP-1 site and the E-box motif are indicated. Cell extracts were assayed for luciferase activity and the -fold induction was determined relative to the control. *, significant differences LPS *versus* control; **, pHO-50 + LPS *versus* pHO-1338 + LPS. (B) HO-1 reporter gene constructs with mutations in the CRE/AP-1 and E-box sites of pHO-754 were generated as described under Materials and Methods. Cell extracts were assayed for luciferase activity and the -fold of induction was determined relative to the control. *, significant differences LPS *versus* control; **, pHO-754Am/Em + LPS *versus* pHO-754 + LPS. *Ctrl*, control.

3.7. Induction of HO-1 gene expression by the NAD(P)H oxidase inhibitor AEBSF in cell cultures of mouse RAW264.7 cells and human PBMC

Gene expression of HO-1 and NAD(P)H oxidase has previously been demonstrated to be increased simultaneously in monocytes of diabetic patients (Avogaro et al., 2003). Since LPS- and TPA-dependent effect have previously been shown to be mediated via activation of the plasma membrane-associated NAD(P)H oxidase in mononuclear phagocytes (Babior, 1999; Park et al., 2004), the regulatory role of the pharmacological NAD(P)H oxidase inhibitor AEBSF on HO-1 gene expression was investigated in more detail.

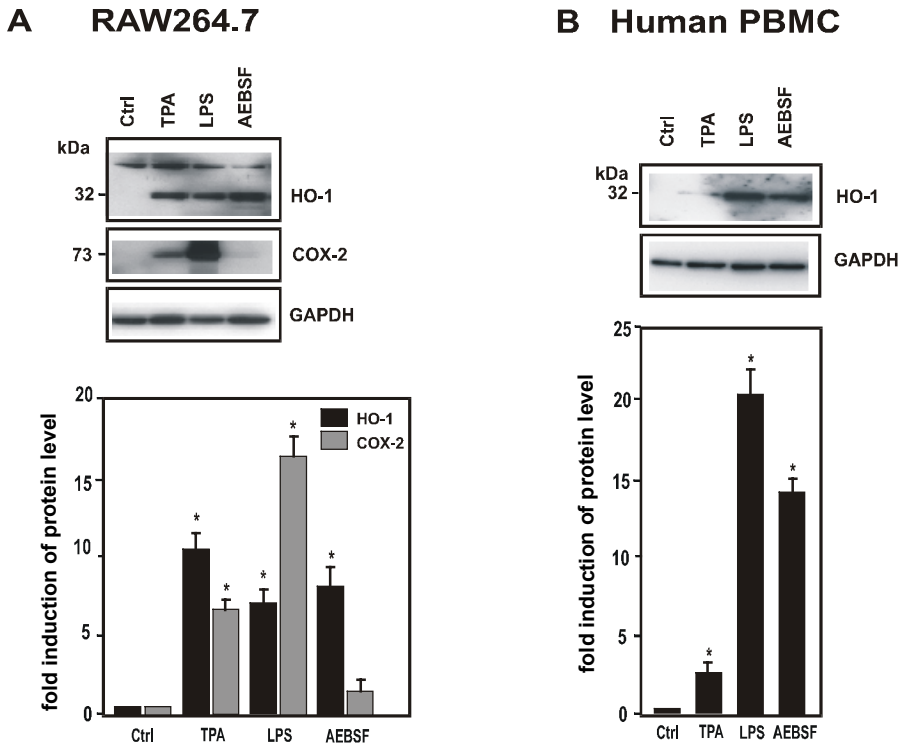

Fig. 12. Induction of HO-1 gene expression by AEBSF in cell cultures of mouse monocytic RAW264.7 cells and human PBMC. Mouse monocytic RAW264.7 cells and human PBMC were cultured as described in Materials and Methods. RAW264.7 cells (A) and human PBMC (B) were treated with TPA (0.5 µM), LPS (1 µg/ml), AEBSF (250 µM) or control medium for 18 h, as indicated. Total protein (30-60 µg) was subjected to Western blot analysis and probed sequentially with antibodies against rat HO-1 and GAPDH. In addition, a COX-2 antibody was applied in (A). *, significant differences AEBSF *versus* control. *Ctrl*, control.

Unexpectedly, treatment of RAW264.7 cells with AEBSF alone led to a marked induction of endogenous HO-1 gene expression (Fig. 12A). AEBSF-dependent induction of HO-1 was also observed in human PBMC (Fig. 12B). The magnitude of the endogenous HO-1 gene induction by AEBSF was in the same range as that observed for LPS and TPA in RAW264.7 cells and that for LPS in human PBMC (Fig. 12). For a comparison, the effects of these stimuli on COX-2 gene expression, which has previously been shown to be induced by LPS and TPA (Smith et al., 2000), was also determined in monocytes.

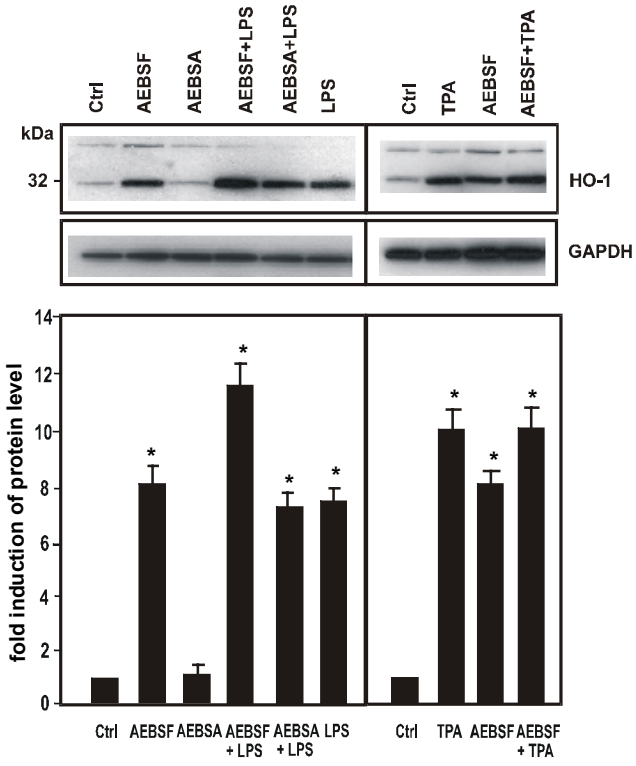

Fig. 13. Effects of AEBSA and combinations of AEBSF with LPS and TPA on endogenous HO-1 gene expression in RAW264.7 cells. RAW264.7 cells were cultured as described in Materials and Methods and were treated with AEBSF (250 µM), AEBSA (250 µM), LPS (1 µg/ml), combinations of AEBSF plus LPS, AEBSA plus LPS, TPA (0.5 µM), AEBSF plus TPA or control medium for 18 h, as indicated. Total protein (60 µg) was subjected to Western blot analysis and probed sequentially with antibodies against rat HO-1 and GAPDH. *, significant differences AEBSF *versus* control. *Ctrl*, control.

In contrast to HO-1, COX-2 gene expression was markedly up-regulated by TPA and LPS, but not by AEBSF in RAW264.7 cells (Fig. 12A). When added in combination with LPS or TPA, AEBSF had an additive effect on the LPS-dependent induction of HO-1 protein

levels and caused no major alteration of HO-1 induction by TPA (Fig. 13). Treatment with 4-(2-aminoethyl) benzenesulfonamide (AEBSA), a homologue of AEBSF in which the sulfonyl fluoride of AEBSF is replaced by an amide group with no inhibitory effect on NAD(P)H oxidase activity (Diatchuk et al., 1997), did not affect the basal or LPS-induced HO-1 gene expression (Fig. 13). The increase of HO-1 protein expression by AEBSF occurred in a time-dependent manner with a maximum after 8 h (Fig. 14) which was earlier than the maximum observed during LPS treatment (see also Fig. 5B).

Moreover, the induction of HO-1 gene expression by AEBSF showed a dose-dependency with a peak dose at 250 µM (data not shown). An increase of HO-1 gene expression by AEBSF was also observed in human umbilical cord endothelial cells, but not in cell cultures of porcine kidney epithelial cells (LLC-PK1) or human cervix epithelial cells (HeLa cells) suggesting a cell-specific mode of HO-1 gene regulation by this compound (data not shown). The data indicate that the NAD(P)H oxidase inhibitor AEBSF is a potent inducer of endogenous HO-1 gene expression in monocytic cells.

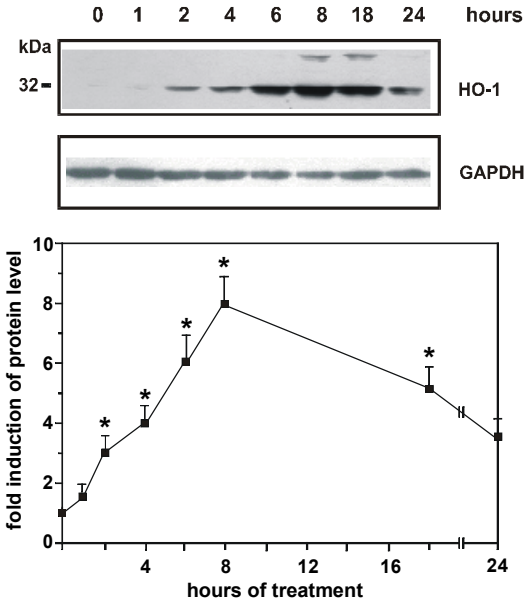

Fig. 14. Time-dependency of HO-1 gene induction by AEBSF. Mouse monocytic RAW264.7 cells were cultured as described in Materials and Methods and were treated with AEBSF (250 µM) for the times indicated. Total protein (60 µg) was subjected to Western blot analysis and probed sequentially with antibodies against rat HO-1 and GAPDH. *, significant differences AEBSF *versus* control.

3.8. Induction of HO-1 promoter activity by AEBSF

To probe into the regulatory mechanism of AEBSF-dependent HO-1 gene induction, RAW264.7 cells were transiently transfected with pHO-1338 which was markedly up-regulated by treatment with AEBSF. The level of AEBSF-dependent induction of HO-1 promoter activity was in a similar range as that observed for LPS, but lower than that for TPA (Fig. 15A). Simultaneous treatment with AEBSF plus LPS had an additive effect on HO-1 promoter activity. For a comparison, the effect of AEBSF was also examined in RAW264.7 cells transfected with reporter gene constructs containing either multiple copies of the consensus recognition sequences for NF-κB (pNF-κB) or AP-1 (pAP-1). Both, NF-κB and AP-1, have previously been shown to be key TFs for LPS- and TPA-mediated gene regulation in mononuclear phagocytes (Guha and Mackman, 2001). No induction by AEBSF was observed for luciferase activity of pAP-1 and pNF-κB (Fig. 15A), but LPS and TPA markedly induced luciferase activity of these reporter gene constructs (Fig. 15A). Remarkably, LPS-dependent induction of constructs pNF-κB and pAP-1 was reduced by simultaneous treatment with AEBSF (Fig. 15A, lower panel). Similarly, the TPA-dependent induction of pNF-κB, but not that of pAP-1 was inhibited by the presence of AEBSF (Fig. 15A, lower panel). No regulatory effect on the promoter activity of pHO-1338 was observed for the AEBSF homologue AEBSA (Fig. 15B) which agrees with the regulation of endogenous HO-1 gene expression (compare with Fig. 13). The data suggest that the AEBSF-dependent induction of HO-1 gene expression is mediated via a transcriptional mechanism which appears to be independent of NF-κB and AP-1.

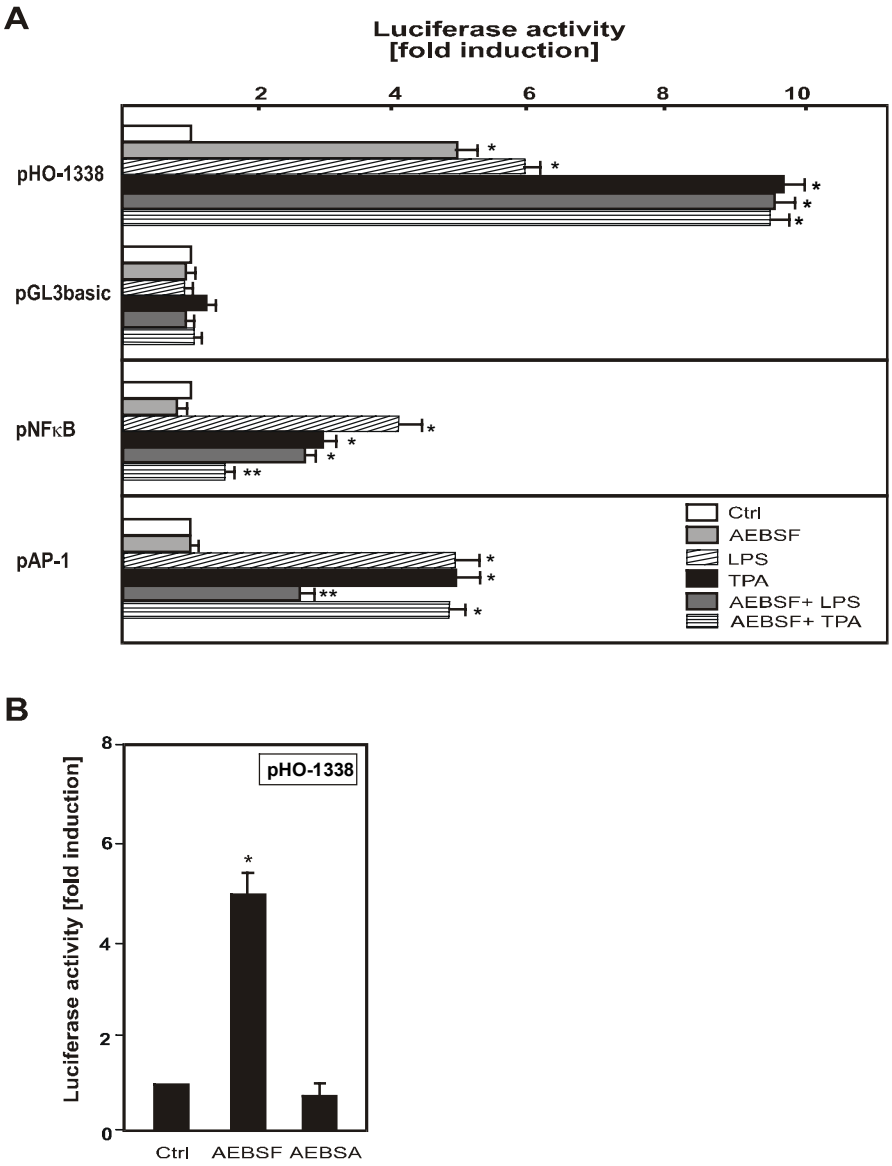

Fig. 15. Induction of rat HO-1 promoter activity by AEBSF in transiently transfected RAW264.7 cells. (A) RAW264.7 cells were transiently transfected with reporter gene constructs containing the proximal 1338 bp of the rat HO-1 gene promoter region (pHO-1338), four copies of the consensus sequence for NF-κB (pNFκB) or three copies of the AP-1 consensus motif (pAP-1) or empty control vector pGL3basic. 24 h after transfection cells were treated for 18 h with or without AEBSF (250 µM), LPS (1 µg/ml), TPA (0.5 µM) or combinations of AEBSF plus LPS and AEBSF plus TPA, as indicated. (B) Cells were transfected with pHO-1338 and 24 h after transfection cells were treated for 18 h with or without AEBSF (250 µM) and AEBSA (250 µM), as indicated. Cell extracts were assayed for luciferase activity and the -fold induction in each experiment relative to the control was determined. *, significant differences treatment *versus* control; **, AEBSF + TPA *versus* TPA and AEBSF + LPS *versus* LPS. *Ctrl,* control.

3.9. Regulatory role of the PI3K/PKB pathway for AEBSF-dependent activation of HO-1 gene expression

The PI3K/PKB signaling pathway has recently been demonstrated to be involved in the induction of HO-1 gene expression by carnosol (Martin et al., 2004) and the 3-hydroxy 3-methylglutaryl coenzyme A reductase inhibitor simvastatin (Lee et al., 2004). To evaluate the regulatory role of this pathway for the AEBSF-dependent induction of HO-1 expression various pharmacological inhibitors were used for pretreatment of RAW264.7 cells. Up-regulation of endogenous HO-1 expression and that of HO-1 promoter activity by AEBSF was markedly reduced by pretreatment with the PI3K inhibitor wortmannin (Fig. 16). Moreover, the PI3K inhibitor LY294002 and the PKB inhibitor SH-5 caused a significant reduction of HO-1 promoter induction by AEBSF (Fig. 16B).

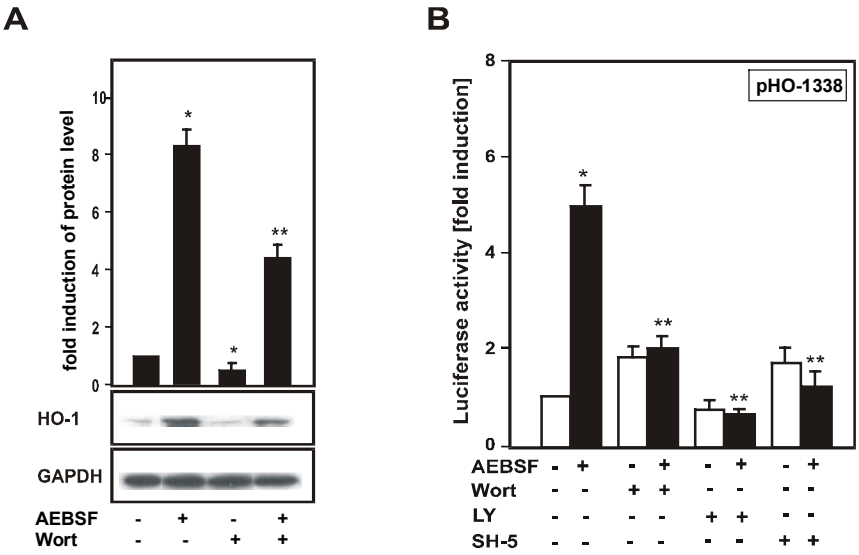

Fig. 16. Inhibition of AEBSF-dependent induction of HO-1 gene expression by PI3K/PKB inhibitors. (A) RAW264.7 cells were pretreated for 1 h with wortmannin (500 nM) before incubation with AEBSF (250 µM). Total protein (40 µg) was subjected to Western blot analysis and probed sequentially with antibodies against rat HO-1 and GAPDH. *, significant differences treatment *versus* control; **, Wort + AEBSF *versus* AEBSF. (B) Cells were transiently transfected with reporter gene construct pHO-1338. 24 h after transfection cells were treated for 1 h with the PI3K inhibitor wortmannin (500 nm), LY294002 (10 µM) or the PKB inhibitor SH-5 (10 µM) before incubation with AEBSF (250 µM) or control medium. After 18 h cell extracts were assayed for luciferase activity and the -fold induction relative to the control was determined. *, significant differences AEBSF *versus* control; **, Wort + AEBSF *versus* AEBSF, LY + AEBSF *versus* AEBSF, SH-5 + AEBSF *versus* AEBSF. *Wort*, wortmannin; *LY*, LY294002.

To verify that AEBSF is an activator of PKB, cell extracts of AEBSF-treated RAW264.7 cells were analyzed for phosphorylated and total PKB. A marked increase of Thr^{308} phosphorylated PKB was observed upon AEBSF treatment (Fig. 17A).

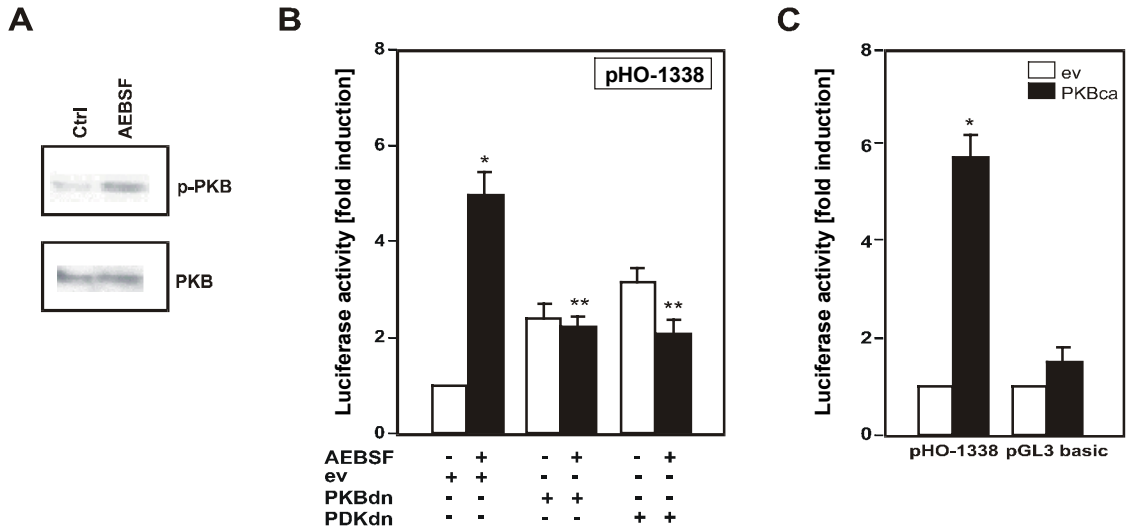

Fig. 17. Role of PKB activation for AEBSF-dependent induction of HO-1 promoter activity. (A) RAW264.7 cells were treated for 1 h with AEBSF and total protein (60 µg) was subjected to Western blot analysis and probed with an antibody for phosphorylated PKB (Thr^{308}). Subsequently, the membrane was stripped and reprobed with an antibody against total PKB. A representative autoradiogram is shown. (B) RAW264.7 cells were cotransfected with pHO-1338, an expression vector for dominant negative PKB, dominant negative PDK or empty expression vector, as indicated. 24 h after transfection cells were treated with or without AEBSF (250 µM) for another 18 h. Cell extracts were assayed for luciferase activity and the -fold induction relative to the control was determined. *, significant difference AEBSF + ev *versus* control; **, AEBSF + PKBdn *versus* AEBSF + ev, AEBSF + PDKdn *versus* AEBSF + ev. (C) RAW264.7 cells were cotransfected with pHO-1338 and empty expression vector or an expression vector for constitutive active PKB, as indicated. 24 h after transfection cell extracts were assayed for luciferase activity and the -fold induction was determined relative to the control. *, significant differences PKBca *versus* control. *ev*, empty vector; *p-PKB*, phospho PKB; *PKBdn*, dominant negative PKB; *PDKdn*, dominant negative PDK; *PKBca*, constitutive active PKB.

To confirm the functional relevance of PKB activation for AEBSF-dependent induction of HO-1 gene expression, the effects of overexpressed dominant negative and constitutive active mutants of PKB were determined. Overexpression of dominant negative PKB slightly increased basal HO-1 promoter activity, but abolished the AEBSF-dependent induction of HO-1 promoter activity (Fig. 17B). Overexpressed dominant negative phosphoinositide-

dependent protein kinase-1 (PDK), an effector molecule of PI3K that targets PKB, also inhibited the AEBSF-dependent activation of HO-1 promoter activity (Fig. 17B). Finally, the specificity of HO-1 gene expression via the PKB pathway was examined by cotransfection of a constitutively active mutant of PKB (myrPKB) which led to a significant up-regulation of HO-1 gene promoter activity (Fig. 17C). The data indicate that the PI3K/PKB signaling cascade is crucially involved in AEBSF-dependent regulation of HO-1 gene expression.

3.10. Role of p38 MAPK for HO-1 gene regulation by AEBSF

To determine the potential regulatory role of MAPKs for the induction of HO-1 gene expression by AEBSF, the phosphorylation of MAPKs was analyzed by Western blot analysis in RAW264.7 cells. Phosphorylation of JNK and p38 was increased in AEBSF-treated cells with a maximum after 30 min, respectively. By contrast, no effect was observed for the level of phosphorylated ERK1/2 in AEBSF-treated cells (Fig. 18). This regulatory pattern of MAPK phosphorylation was different from that observed for LPS in RAW 264.7 cells (for comparison see Fig. 9).

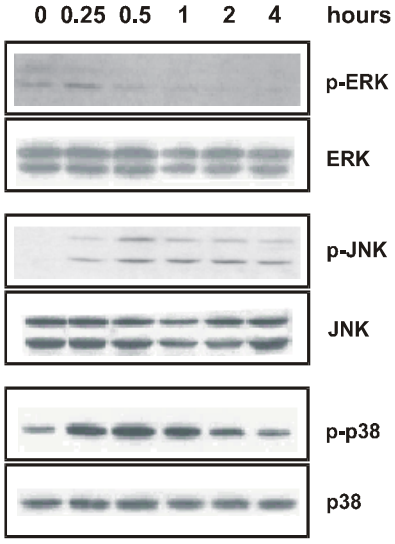

Fig. 18. Activation of MAPKs by AEBSF in RAW264.7 cells. RAW264.7 cells were cultured as described in Materials and Methods and were treated with AEBSF (250 µM) for the times indicated. Total protein (60 µg) was subjected to Western blot analysis and probed with antibodies for various MAPKs. Membranes were initially used to detect phosphorylated MAPKs, then stripped and probed with antibodies against total MAPKs. Autoradiograms of a representative experiment are shown, respectively. *p-ERK*, phospho ERK; *p-JNK*, phospho JNK; *p-p38*, phospho p38.

Next, the effects of specific MAPK inhibitors on the AEBSF-dependent regulation of the endogenous HO-1 gene expression was examined. The p38 inhibitor SB203580, but not the ERK inhibitor PD98059 nor the JNK inhibitor SP600125, reduced the AEBSF-dependent induction of HO-1 protein expression (Fig. 19).

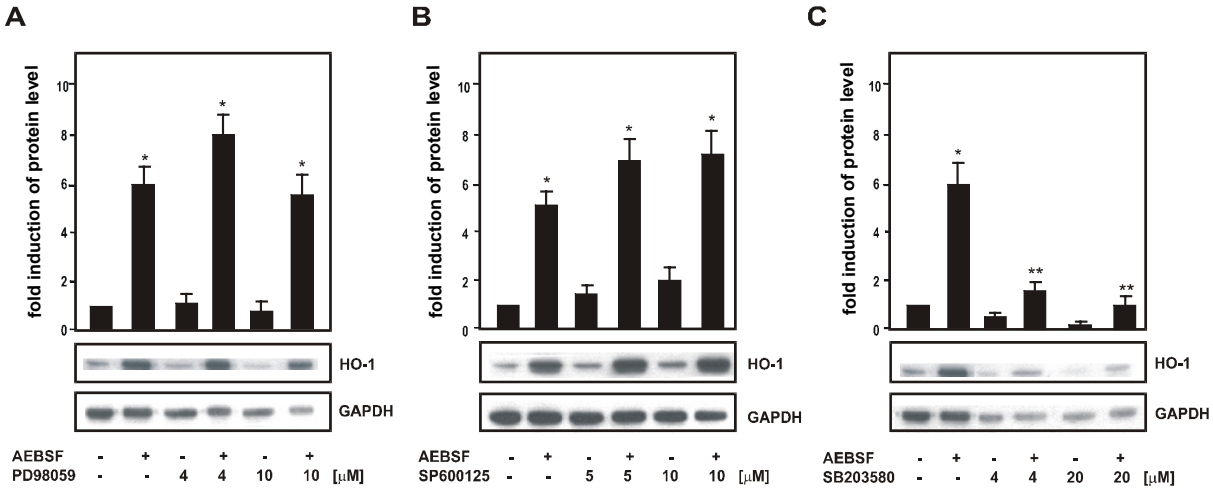

Fig. 19. Effect of MAPK inhibitors on AEBSF-dependent induction of HO-1 gene expression. (A-C) RAW264.7 cells were pretreated for 1 h with various inhibitors of MAPKs at the indicated concentrations. Thereafter, cells were incubated for another 18 h in the absence or presence of AEBSF (250 μM), as indicated. Total protein (60 μg) was analyzed by Western blot analysis with antibodies against rat HO-1 and GAPDH. Autoradiograms of a representative experiment are shown, respectively. *, significant differences treatment *versus* control; **, SB203580 + AEBSF *versus* AEBSF.

To investigate the role of MAPKs for the transcriptional regulation of the HO-1 gene by AEBSF, the effects of MAPK inhibitors on HO-1 promoter activity were determined. While inhibitors of the ERK and JNK pathways, PD98059 and SP600125, had no effects, respectively (Fig. 20A), the p38 inhibitors SB203580 and SB202190 abolished AEBSF-dependent induction of HO-1 promoter activity (Fig. 20B). The regulatory role of p38 for AEBSF-dependent HO-1 gene induction was further delineated by cotransfection of expression vectors with dominant negative mutants (AF) of the p38α, β, γ and δ isoforms. Overexpression of dominant negative p38α and p38β significantly reduced the induction of HO-1 promoter activity by AEBSF, but dominant negative forms of p38γ and p38δ had only minor inhibitory effects (Table 4). Taken together, the data suggest that p38α and p38β play a major regulatory role for the induction of HO-1 gene expression by AEBSF.

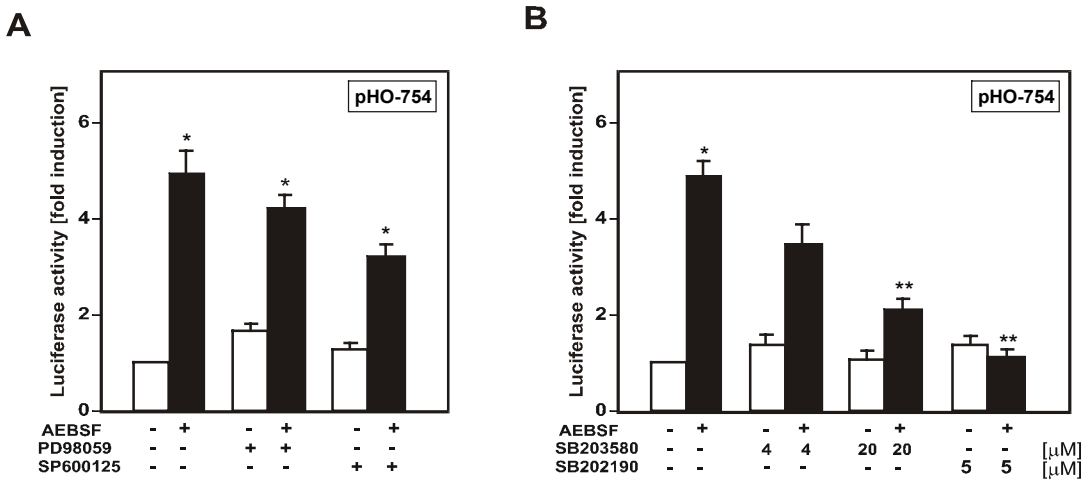

Fig. 20. Effect of MAPK inhibitors on AEBSF-dependent induction of HO-1 promoter activity. RAW264.7 cells were transfected with the HO-1 reporter gene construct pHO-1338. 24 h after transfection cells were pretreated with the MAPKs inhibitors (A) PD98059 (4 μM), or SP600125 (10 μM), or (B) SB203580, and SB202180 at the indicated concentrations for 1 h. Then, cells were incubated for another 18 h in the absence or presence of AEBSF (250 μM). Cell extracts were assayed for luciferase activity and the -fold induction relative to the control was determined. *, significant differences AEBSF *versus* control; **, SB203580 + AEBSF *versus* AEBSF, SB202190 + AEBSF *versus* AEBSF.

Table 4. Comparative effects of overexpressed dominant negative p38 isoforms on AEBSF-dependent induction of HO-1 promoter activity. RAW264.7 cells were cotransfected with pHO-1338 and dominant negative mutants (AF) for p38α, β, γ and δ or empty expression vector. After 18 h cell culture was continued in the presence of AEBSF or control medium. Values show the fold induction of luciferase activity relative to control. *, significant differences p38αdn + AEBSF *versus* ev + AEBSF, p38βdn + AEBSF *versus* ev + AEBSF.; *ev*, empty vector; *p38αdn*, dominant negative p38α; *p38βdn*, dominant negative p38β; *p38γdn*, dominant negative p38γ; *p38δdn*, dominant negative p38δ.

Cotransfected plasmid	fold induction of luciferase activity by AEBSF
empty vector	4.5 ± 0.5
p38αdn	1.3 ± 0.2*
p38βdn	1.2 ± 0.1*
p38γdn	2.5 ± 0.2
p38δdn	3.2 ± 0.4

3.11. p38 is a downstream target of AEBSF-dependent PKB activation

To find out whether PKB is required for AEBSF-dependent stimulation of p38 MAPK, the activation of p38 was determined with a fusion plasmid containing the transactivation domain of the TF CHOP and the DNA-binding domain of yeast Gal4 (pFA-CHOP). Transactivation via pFA-CHOP is specifically controlled by p38-dependent phosphorylation of two adjacent regulatory serine residues of the CHOP transactivation domain (Wang and Ron, 1996) which can be monitored via a cotransfected Gal4 luciferase reporter gene construct (pGal4-luc). Treatment with AEBSF induced activity of pFA-CHOP to a similar level as that for the known p38 activator TPA (Fig. 21). AEBSF-dependent induction of pFA-CHOP was reduced by pre-treatment with the PKB inhibitor SH-5 and overexpression of dominant negative PKB. As expected, up-regulation of pFA-CHOP-mediated luciferase activity was inhibited by the p38 inhibitor SB202190. The data suggest that AEBSF-dependent activation of PKB is required for activation of p38 in RAW264.7 cells.

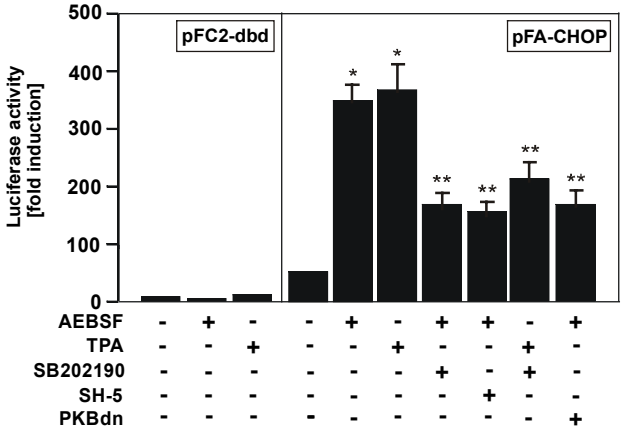

Fig. 21. Regulation of AEBSF-dependent induction of CHOP transactivity by inhibiton of p38 MAPK and PKB. RAW264.7 cells were cotransfected with luciferase reporter gene construct pGal4-luc, pFC2-dbd, pFA-CHOP and an expression vector for dominant negative PKB, as indicated. 24 h after transfection cells were treated with AEBSF (250 µM), TPA (0.5 µM), SB202190 (2.5 µM) or PKB inhibitor SH-5 (20 µM), as indicated. Cell extracts were assayed for luciferase activity and the -fold induction relative to the control was determined. *, significant differences treatment *versus* control; **, SB202190 + AEBSF *versus* AEBSF, SH-5 + AEBSF *versus* AEBSF, SB202190 + TPA *versus* TPA, PKBdn + AEBSF *versus* AEBSF. *PKBdn*, dominant negative PKB.

3.12. Role of the E-box and CRE/AP-1 elements for AEBSF- and PKB-dependent induction of HO-1 promoter activity

To identify (the) *cis*-acting RE(s) that mediate(s) the AEBSF-dependent induction of HO-1 gene expression, various HO-1 promoter gene constructs were transiently transfected into RAW264.7 cells. Deletion of the rat HO-1 gene promoter sequence from -1338 to -754 did not affect the responsiveness of reporter gene activity by AEBSF (pHO-754; Fig. 22A). Neither of two reporter gene constructs with a mutation of the E-box site (-47 to -42; pHO-754Em) or the CRE/AP-1 site (-668 to -654; pHO-754Am) showed a lower responsiveness to AEBSF. By contrast, simultaneous mutation of the E-box site and the CRE/AP-1 element (pHO-754Am/Em) led to a marked reduction of AEBSF-mediated induction of reporter gene activity (Fig. 22A).

The regulation of these HO-1 reporter gene constructs was also examined in cells that were cotransfected with an expression vector for constitutive active PKB. Similar to the regulatory pattern of reporter gene activity by AEBSF, the overexpression of a constitutive active PKB mutant induced luciferase activity of wild type pHO-754, but not that of pHO-754Am/Em (Fig. 22B). No appreciable reduction of PKB-mediated responsiveness was observed for the HO-1 promoter gene constructs with either a mutation of the E-box or the CRE/AP-1 site (data not shown). These findings suggest that the AEBSF- and PKB-dependent activation of HO-1 gene expression is mediated via a transcriptional mechanism that involves the E-box and the CRE/AP-1 element of the proximal HO-1 promoter region.

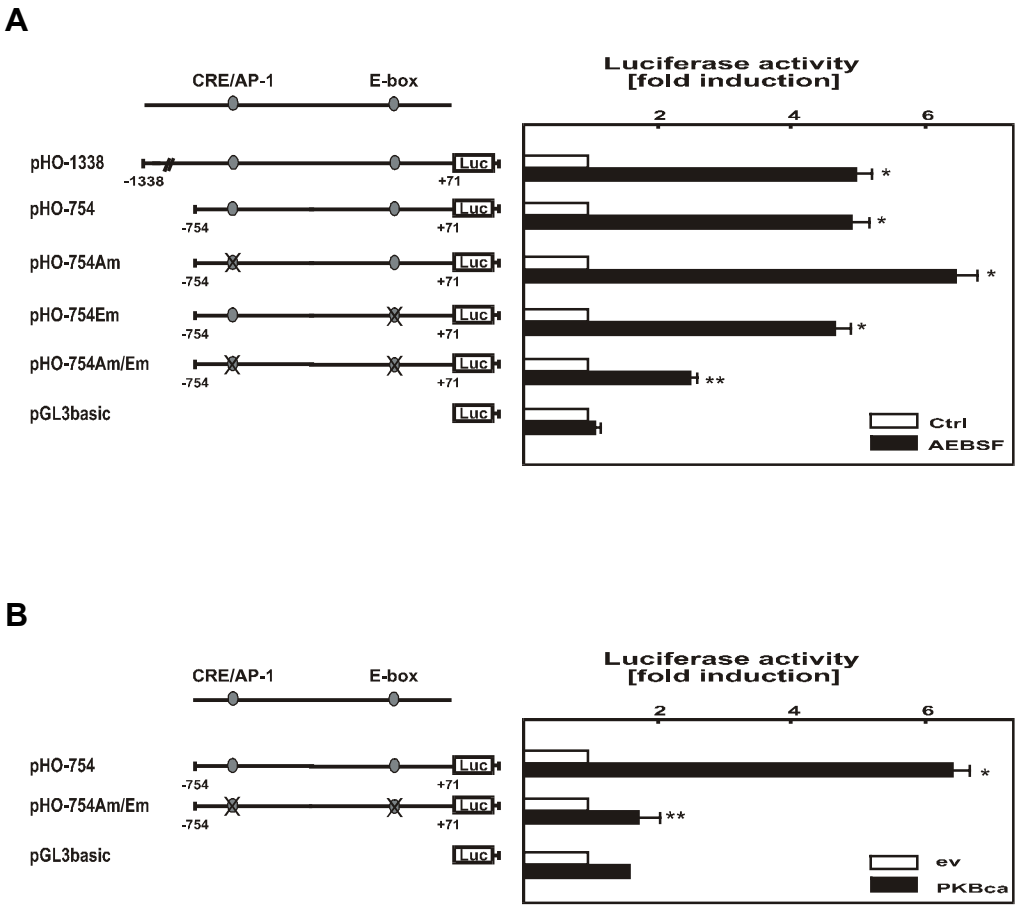

Fig. 22. Regulation of various rat HO-1 promoter gene constructs by AEBSF and overexpressed constitutive active PKB. (A) HO-1 reporter gene constructs with the indicated mutations of the E-box and the CRE/AP-1 element of the rat HO-1 promoter were transfected into RAW264.7 cells. 24 h after transfection cells were treated for 18 h with control medium or medium supplemented with AEBSF (250 µM). Cell extracts were assayed for luciferase activity, and the -fold induction was determined relative to the control. *, significant differences treatment *versus* control; **, pHO-754Am/Em + AEBSF *versus* pHO-754 + AEBSF. (B) Cells were cotransfected with the indicated HO-1 reporter gene constructs and empty expression vector or an expression vector for constitutive active PKB, as indicated. 24 h after transfection cell extracts were assayed for luciferase activity and the -fold induction was determined relative to the control. *, significant differences PKBca *versus* control; **, pHO-754Am/Em + PKBca *versus* pHO-754 + PKBca. Ctrl, control; ev, empty vector; *PKBca*, constitutive active PKB.

3.13. Role of USF2 and coactivator p300 for the AEBSF-dependent induction of HO-1 promoter activity

The identification of a functional E-box motif in the HO-1 promoter along with the previous findings that basic helix-loop-helix TFs may serve as nuclear targets for p38 (Galibert et al., 2001) suggest that USF2 could be involved in the AEBSF-dependent induction of HO-1 gene expression. Therefore, cotransfection experiments were performed with expression vectors for wild type USF2 and a dominant negative mutant of USF (ΔbTDU1) which dimerizes with USF proteins, but lacks the DNA-binding and transactivation domain (Lefrancois-Martinez et al., 1995). The AEBSF-dependent responsiveness of HO-1 reporter gene activity was enhanced by overexpressed wild type USF2 and was reduced by overexpression of the ΔbTDU1 mutant (Fig. 23).

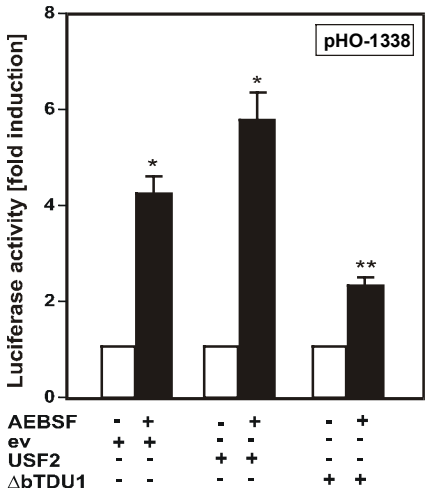

Fig. 23. Effect of overexpressed USF2 on AEBSF-dependent induction of HO-1 promoter activity. RAW264.7 cells were cotransfected with pHO-1338 and expression vectors for either wild-type USF2 or dominant negative USF2 (ΔbTDU1) or an empty control expression vector, as indicated. 24 h after transfection cells were treated for another 18 h with or without AEBSF (250 μM). Cell extracts were assayed for luciferase activity and the -fold induction relative to the control was determined. *, significant differences AEBSF *versus* control; **, ΔbTDU1 + AEBSF *versus* control + AEBSF. *Ctrl*, control; *ev*, empty vector.

USF2 has recently been found to recruit the transcriptional coactivator p300/ CREB-binding protein (CBP) (Goueli and Janknecht, 2003; Blobel, 2002). Therefore, the effect of a cotransfected expression vector for p300 on reporter gene activity of wild type pHO-754 and that of pHO-754Am/Em with targeted mutations of the E-box and the CRE/AP-1 sites in RAW264.7 cells was examined. Overexpression of p300 strongly increased the basal and AEBSF-dependent induction of pHO-754 promoter activity (Fig. 24). In contrast, overexpression of p300 only had a minor effect on the basal and AEBSF-augmented promoter activity of pHO-754Am/Em. No effect of cotransfected p300 was observed for luciferase activity of control vector pGL3basic (Fig. 24). Taken together, the data suggest that USF2 and p300 are involved in the transcriptional induction of HO-1 gene expression by AEBSF in RAW264.7 cells.

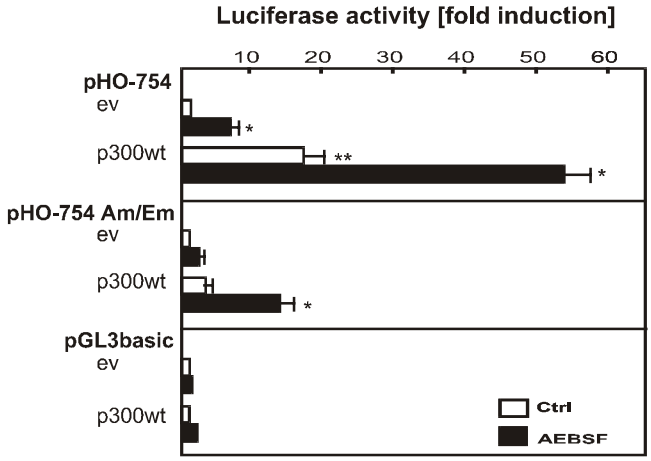

Fig. 24. Effect of overexpressed p300 on AEBSF-dependent induction of HO-1 promoter activity. RAW264.7 cells were cotransfected with reporter gene constructs pHO-754, or pHO-754Am/Em and an expression vector for p300 or empty expression vector. 24 h after transfection cells were treated for 18 h with or without AEBSF (250 µM). Cell extracts were assayed for luciferase activity and the fold induction relative to the control was determined. *, significant differences AEBSF *versus* control; **, p300 *versus* ev. *Ctrl*, control; *ev*, empty vector.

4. DISCUSSION

The HO-1 gene codes for the first and rate-limiting enzyme of heme degradation and is induced by multiple stress stimuli. Overexpression of the HO-1 gene not only plays a protective role against oxidant damage of cells and tissues, but also modulates the inflammatory immune response. The major findings of the present study are that the inflammatory mediator LPS and the NAD(P)H-oxidase inhibitor AEBSF induce HO-1 gene expression in cell cultures of mouse monocytes by a transcriptional mechanism that involves *cis*-acting REs of the proximal rat HO-1 gene promoter. Activation of the NF-κB-, PKB- and p38 MAPK-signaling pathways is involved in the LPS- and AEBSF-dependent induction of HO-1 gene expression.

4.1. Induction of HO-1 gene expression by LPS and the NAD(P)H oxidase inhibitor AEBSF in monocytes

The prototypical inflammatory stimulus LPS induced HO-1 gene expression in our experimental model of RAW264.7 monocytes (Fig. 5) which agrees with previous reports (Camhi et al., 1995, 1998, Immenschuh et al., 1999). By contrast, the up-regulation of HO-1 gene expression by the NAD(P)H oxidase inhibitor AEBSF was unexpected (Fig. 12) because LPS-dependent activation of NAD(P)H oxidase is mediated by direct interaction of the TLR4 with this enzyme (Park et al., 2004) and AEBSF has previously been shown to inhibit the enzymatic generation of ROS by NAD(P)H oxidase (Diatchuk et al., 1997). Therefore, it was postulated that pretreatment of monocytes with AEBSF would down-regulate not only the induction of HO-1 by LPS, but also that by the phorbol ester TPA, which is a potent inducer of the membrane-associated NAD(P)H oxidase (Babior, 1999). As demonstrated in Fig. 13 the opposite was true and the up-regulation of HO-1 gene expression by LPS was increased by simultaneous treatment of monocytes with AEBSF plus LPS. Moreover, the induction of HO-1 gene expression by TPA was not markedly affected by treatment with AEBSF (Fig. 13). Remarkably, the induction of HO-1 gene expression by AEBSF was not restricted to mouse RAW264.7 cells, but was also observed in cultured human PBMC (Fig. 12B). This finding may suggest that AEBSF-dependent HO-1 induction may be of physiological significance in mononuclear phagocytes. The induction of HO-1 gene expression by AEBSF appears to be specific, because no regulatory effect of this compound was observed for COX-2 gene expression, which is known to be induced by a

number of stimuli that also up-regulate the HO-1 gene, including LPS and TPA (Fig. 12A). The unexpected observation that AEBSF is an inducer of HO-1 may be similar to the up-regulation of HO-1 gene expression by the antioxidant compounds pyrrolidine dithiocarbamate, caffeic acid phenethyl ester or carnosol (Hartsfield et al., 1997; 1998; Scapagnini et al., 2002; Martin et al., 2004). The antioxidant and metal-chelator pyrrolidine dithiocarbamate has previously been demonstrated to be a potent inducer of HO-1 gene expression in monocytes (Hartsfield et al., 1997; 1998). More recently, caffeic acid phenethyl ester and the herb-derived diterpene carnosol were shown to increase HO-1 gene expression in astrocytes and rat pheochromocytoma cells (Scapagnini et al., 2002; Martin et al., 2004), respectively. Remarkably, the time courses of endogenous HO-1 gene expression in RAW264.7 cells in response to LPS and AEBSF were different suggesting that distinct mechanisms mediate the induction of HO-1 gene expression by these compounds (compare Figs. 5B and 14).

4.2. Transcriptional mode of HO-1 induction by LPS and AEBSF and identification of *cis*-acting REs of the proximal rat HO-1 promoter region

To further investigate the regulatory mechanisms that are involved in the induction of HO-1 gene expression by LPS and AEBSF, transfection experiments with reporter gene constructs of the rat HO-1 gene promoter were performed in RAW264.7 monocytes.

REs for the LPS-dependent HO-1 gene induction

It has been demonstrated in earlier reports that the LPS-dependent induction of HO-1 gene expression is mediated by two far upstream regions localized at -4 kb and -12 kb relative to the transcription initiation site of the mouse HO-1 promoter, respectively (see also Fig. 3). Since both regions contain several potential AP-1 recognition sequences, it has initially been assumed that members of the AP-1 TF family would interact with these two HO-1 promoter regions and would mediate stress-dependent HO-1 activation such as that elicited by LPS (Alam, 1994; Alam et al., 1995; Camhi et al., 1998). In a follow-up study, however, it turnt out that these two promoter regions were nuclear targets for the stress-regulated TF Nrf2 (Alam et al., 1999). Nrf2 is a prototypical regulator for oxidative stress stimuli such as electrophilic substances and glutathione-depleting compounds (for a review see Nguyen et al., 2003). It is well known that NF-κB plays an important role for the induction of numerous genes by LPS in mononuclear phagocytes (Guha and Mackman, 2001).

Therefore, it was first hypothesized that a putative NF-κB site of the rat HO-1 promoter (-1002/-994), which matches the prototypical NF-κB consensus sequence in 10 out of 11 bp, may be involved in the transcriptional induction of LPS-dependent HO-1 gene induction. This putative NF-κB site, however, did not play a functional role for HO-1 induction by LPS as demonstrated by transfection studies with various luciferase reporter gene constructs (Fig. 11A). The data rather suggest that (a) RE(s) of the proximal promoter localized between -347 and -50 may mediate the LPS-dependent induction of HO-1 gene expression, because a major reduction of LPS-dependent HO-1 promoter induction was observed after deletion of this sequence (Fig. 11A). Similar to the findings of the present study, it was recently demonstrated by Chung et al. (2005) that two recognition sequences for the TF Ets (-125 to -121 and -93 to -88) of the proximal HO-1 promoter played a crucial role for HO-1 induction by LPS in monocytes. The authors have shown that deletion of these Ets binding sites led to a complete loss of LPS-dependent induction of HO-1 promoter activity in transfected RAW264.7 cells. Moreover, it was shown that overexpression of wild type Ets increased, but overexpression of dominant negative Ets blunted the LPS-dependent induction of HO-1 promoter activity. Taken together, the data of the present study along with those of Chung et al. (2005) demonstrate that not only the distal promoter region of the HO-1 gene, but also the proximal promoter region of the rodent HO-1 gene plays an important role for the regulation of HO-1 gene expression by LPS and, possibly, also by other stress genes.

REs for the AEBSF-dependent HO-1 gene induction

In contrast to the transcriptional HO-1 induction by LPS, the responsiveness of luciferase reporter gene constructs with serial deletions of the rat HO-1 gene promoter to AEBSF showed no significant reduction (data not shown). It is also remarkable that reporter gene constructs with target sequences for NF-κB and AP-1, both of which are known to mediate the gene regulation by LPS and TPA, did not respond to treatment with AEBSF (Fig. 15). Simultaneous deletion of the E-box (-47 to -42) and the CRE/AP-1 element (-664 to -657) of the proximal HO-1 promoter region, however, significantly reduced the AEBSF-dependent induction of HO-1 reporter gene activity (Fig. 22A). The identification of a functional E-box motif in the HO-1 promoter suggested that basic helix-loop-helix TF such as USF2 may be involved in the AEBSF-dependent induction of HO-1 gene expression. In fact, the data on the regulatory effects of overexpressed wild type or dominant negative USF2 supported this notion (Fig. 23). The observations may agree with a previous report in which the proximal E-box motif of the human HO-1 gene promoter was demonstrated to play a

major regulatory role for the phorbol ester-dependent HO-1 gene regulation in the monocytic cell line THP-1 (Muraosa et al., 1993). In addition, it has recently been reported that USF2 mediated basal and inducible HO-1 gene expression in renal proximal tubular cells via physical interaction with the proximal E-box of the human HO-1 gene as demonstrated by in vivo footprinting assays (Hock et al., 2004). It is also conceivable, however, that other TFs that target E-box sequences, may interact with the proximal HO-1 E-box such as the TFs Myc and Max. A regulatory role of Max via interaction with the proximal rat HO-1 E-box has previously been demonstrated for HO-1 gene induction by sodium arsenite in primary rat hepatocytes (Kietzmann et al., 2003).

The data of the present study also indicate that p300, which is also known as CREB-binding protein (CBP), is involved in AEBSF-dependent HO-1 gene induction. This mechanism appears to involve a cooperative interaction of the E-box and the CRE/AP-1 element of the HO-1 promoter (Fig. 24). Since p300 mediates cooperative interaction of transcriptional regulators via protein-protein contacts and links TFs with components of the basal transcription machinery (Blobel, 2002), it is conceivable that interaction of p300, USF2 and AP-1 may participate in this regulatory mechanism (see scheme Fig. 26). Such a conclusion is underscored by the observation that overexpression of p300 markedly enhanced reporter gene activity of a construct with the wild type HO-1 gene promoter sequence (pHO-754), but not that of a construct with mutations of the E-box and the CRE/AP-1 sites (pHO-754Am/Em; Fig. 24). A cooperative mode of gene regulation via a p300/CBP-dependent mechanism has previously been shown for the epidermal growth factor-dependent up-regulation of keratin 16 gene expression by interaction with the TFs SP-1 and AP-1 (Wang and Chang, 2003) and for the insulin-dependent induction of glucokinase gene expression by interaction with hypoxia-inducible factor-1 and hepatocyte nuclear factor-4α (Roth et al., 2004).

4.3. Signaling pathways that mediate the LPS- and AEBSF-dependent induction of HO-1 gene expression in monocytes

Induction of HO-1 gene expression by LPS and AEBSF is mediated by distinct signaling cascades in RAW264.7 cells. Up-regulation of HO-1 by either stimulus, however, is at least in part, mediated via activation of the common downstream MAPK p38 (summarized in Figs. 25 and 26).

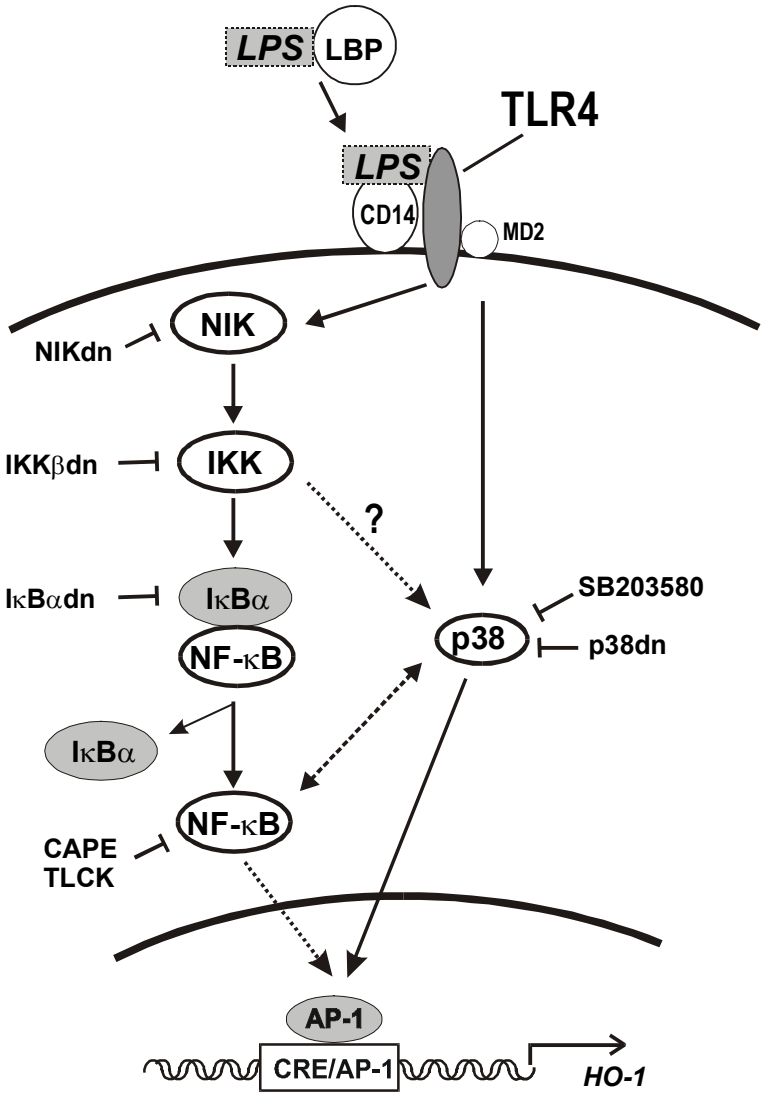

Fig. 25. Signaling pathways of LPS-dependent induction of HO-1 gene expression

LPS-dependent activation of multiple intracellular signaling cascades is mediated via the TLR4. A central player of TLR4-mediated signaling is NF-κB (for a scheme see Fig. 25). The fact that two pharmacological inhibitors of NF-κB (Fig. 7) and dominant negative mutants of kinases of the NF-κB signaling pathway (Fig. 8) inhibit the LPS-dependent induction of HO-1 promoter activity, strongly suggests that NF-κB is involved in LPS-dependent induction of HO-1 gene expression. The nuclear target of this signaling cascade that activates the HO-1 promoter, however, is not entirely clear from the present study. A putative NF-κB recognition sequence of the HO-1 promoter (-1002/-994), which could be a nuclear target of

this pathway, does not seem to play a role for the induction of the HO-1 gene expression (Fig. 9A). Interestingly, it has recently been demonstrated that NF-κB is a crucial upstream regulator of AP-1 proteins such as JunB, JunD and B-ATF (Krappmann et al., 2004). The master regulator within this network is the IKK complex which is responsible for activation and repression of various early immediate genes. Since AP-1 has previously been shown to be involved in the regulation of the HO-1 gene via interaction with the CRE/AP-1 site of the proximal promoter region (Immenschuh et al., 2000; Kietzmann et al., 2003), it is conceivable that an IKK/NF-κB-dependent pathway may be involved in the regulatory mechanism described in the present study. Moreover, additional pathways other than the NF-κB cascade could be involved in the LPS-dependent induction of HO-1 gene expression (for a review see Guha and Mackman, 2001). The possibility that an autocrine mechanism involving the cytokine TNFα may participate in LPS-dependent induction of HO-1 as previously suggested by others (Oguro et al., 2002), is not likely in our experimental system, because treatment with recombinant TNFα did not affect HO-1 gene expression in RAW264.7 cells (Fig. 5C). Alternatively, cross-talk of the NF-κB pathway with other upstream signaling cascades, could be involved in LPS-dependent activation of HO-1. As an example, Irie and colleagues have previously shown that LPS-dependent induction of NF-κB is mediated via the MAPK/ERK kinase kinase, transforming growth factor-β activated kinase-1 (Irie et al., 2000).

The present data clearly demonstrate that activation of the p38 MAPK signaling pathway is involved in the induction of HO-1 gene expression by LPS in RAW264.7 cells (Fig. 9). Overexpression of dominant negative mutants of p38β and p38δ had opposing effects on the LPS-dependent induction of HO-1 gene expression in RAW264.7 cells (Fig. 10B, C) suggesting that various isoforms of p38 play different regulatory roles for LPS-mediated gene regulation. These findings are similar to a recent report, in which AP-1-mediated gene regulation has been shown to be modulated in a highly specific manner by various p38 isoforms in human breast cancer cells (Pramanik et al., 2003). Clearly, the potential interaction of the NF-κB and p38 signaling pathways for the regulation of HO-1 gene expression remains to be clarified. Saccani et al. (2002) have previously demonstrated that recruitment of NF-κB to a subset of inflammatory genes in response to microbial products is mediated via the p38 pathway. These authors have shown that not direct DNA-binding of NF-κB mediated this regulatory mechanism, but modification of the accessibility to NF-κB binding sites in a set of NF-κB-inducible genes was modulated via changes to phosphorylation and acetylation of histone H3 (Saccani et al., 2002). Independently, a complex interplay of upstream signaling cascades has been demonstrated to be involved in

the NF-κB-dependent gene regulation of the monocyte chemoattractant protein-1 gene (Goebeler et al., 2001). Moreover, it has recently been demonstrated by Yang et al. (2003) in mouse embryo fibroblasts from IKKβ-deficient animals that this kinase plays a regulatory role which is independent of direct transcriptional activation. Finally, it has been demonstrated that p38-dependent phosphorylation of the TF TATA-binding protein by LPS is necessary for the activation of NF-κB-dependent gene regulation (Carter et al., 2001). Clearly, further studies are necessary to elucidate the potential cross-talk of these signaling cascades in the context of HO-1 gene regulation.

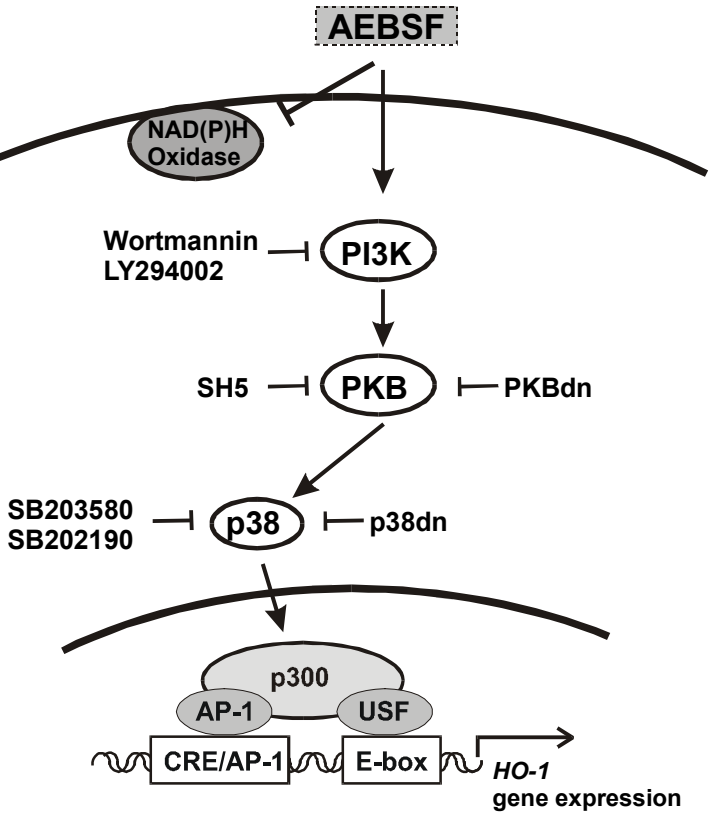

Fig. 26. Signaling pathways of AEBSF-dependent induction of HO-1 gene expression

Since LPS-dependent activation of NAD(P)H oxidase is mediated by direct interaction of TLR4 with this enzyme (Park et al., 2004), it was postulated that treatment with AEBSF would inhibit the LPS-dependent HO-1 gene induction. Unexpectedly, however, an opposite

regulatory pattern of HO-1 gene expression was observed in cell cultures of RAW264.7 cells and human PBMC. Simultaneous treatment of AEBSF plus LPS lead to an additive induction of endogenous HO-1 gene expression and promoter activity (Figs. 13, 15A). The reduction of AEBSF-dependent HO-1 gene induction by pharmacological inhibitors of the PI3K/PKB pathway and overexpression of dominant negative mutants of PKB indicated that the serine/ threonine kinase PKB plays a major role for the AEBSF-dependent activation of the HO-1 gene in monocytes (Figs. 16, 17). The significance of PKB activation for HO-1 gene regulation was confirmed by the finding that HO-1 promoter activity was highly induced by overexpressed constitutively activated PKB (Fig. 17). A regulatory role of the PKB pathway for the induction of HO-1 gene expression has recently been demonstrated in other cell culture systems. PKB has been shown to mediate the HO-1 gene up-regulation by the 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor simvastatin in human and rat vascular smooth muscle cells (Lee et al., 2004). Independently, the phytochemical carnosol has been shown to activate HO-1 gene expression in a PKB-dependent manner in PC12 pheochromocytoma cells (Martin et al., 2004).

The downstream targets of PKB, however, remained elusive in these earlier reports. The present work shows that PKB is required for the activation of p38 MAPK by AEBSF (Fig. 21). It is demonstrated in a Gal4 luciferase assay that the AEBSF-dependent induction of a specific p38 target construct (Gal4-CHOP; Wang and Ron, 1996) is inhibited by treatment with the PKB inhibitor SH-5 and by overexpressed dominant negative PKB (Fig. 21). Therefore, p38 MAPK appears to be a downstream target of PKB in RAW264.7 monocytes. The inhibitory effects of specific pharmacological MAPK inhibitors and various isoforms of dominant negative mutants of p38 indicated that p38α and p38β, but not ERK and JNK, are involved in AEBSF-dependent induction of HO-1 gene expression (Figs. 19, 20, Table 4). Contradictory findings on the potential cross-talk between the PKB and p38 signaling pathways have previously been reported by others. In agreement with the present findings, Lee et al. (2004) have demonstrated that stimulation of p38 MAPK by anisomycin was attenuated by inhibition of the PKB pathway in a follicular dendritic cell line suggesting that PKB is necessary for the activation of p38 MAPK. In contrast, others have shown that p38 was necessary for the MAPK-activated protein kinase-2-dependent phosphorylation of PKB in human neutrophils concluding that p38 is a functional phosphoinositide-dependent kinase-2 for PKB (Rane et al., 2001). In accordance with this report Taniyama and colleagues have demonstrated in vascular smooth muscle cells that the p38 MAPK pathway mediated angiotensin-dependent activation of PKB (Taniyama et al., 2004). More recently, it has been

demonstrated in a model of mouse myoblast differentiation that the p38 and PI3K/PKB pathways are functional in a reciprocal manner. In this elegant study it has been shown that inhibition of p38 reduced PKB activity and that the down-regulation of PI3K/ PKB decreased p38 MAPK activity (Gonzales et al., 2004).

4.4. Physiological functions of HO-1

Major functions of HO enzyme activity comprise degradation of the prooxidant heme and production of CO and bilirubin, thereby providing protection of organs and tissue against oxidative stress (Abraham et al., 1988; Maines, 1997). More recently, accumulating evidence indicates that HO-1 is an important modulator of the inflammatory response possibly via the generation of the second messenger gas CO (Otterbein et al., 2000; 2003). An anti-inflammatory function of HO-1 has been shown in experimental models of acute complement-dependent pleurisy and heme-induced inflammation of various organs (Willis et al., 1996; Wagener et al., 2001). In addition, HO-1 deficient animals have been reported to be highly susceptible to endotoxin-mediated toxicity and to exhibit an immune phenotype that is associated with an exaggerated activation of mononuclear phagocytes (Poss and Tonegawa, 1997; Kapturczak et al., 2004). These observations in HO-1 knockout mice were essentially confirmed in a case of human genetic HO-1 deficiency (Yachie et al., 1999) in which similar phenotypical alterations were observed (Table 3). A potential mechanism that may be involved in HO-1 mediated anti-inflammatory protection could be the regulation of adhesion molecules. Adhesion molecules are important for the recruitment of inflammatory cells in acute and chronic inflammation and it has been demonstrated in several inflammation models that inhibition of HO activity increases the expression of adhesion molecules *in vitro* and *in vivo* (Hayashi et al., 1999; Wagener et al., 1999; Vachharajani et al., 2000; Wagener et al., 2001).

Since LPS-dependent activation of monocytic cells plays a crucial role in the pathogenesis of sepsis, the present data may have important physiological and therapeutic implications. The modification of HO-1 gene expression in monocytic cells via targeted regulation of specific signaling cascades may ultimately lead to the development of novel therapeutic strategies (Immenschuh and Ramadori, 2000). Moreover, the specific up-regulation of the HO-1 gene in monocytes by compounds such as AEBSF may provide novel pharmacological approaches for the treatment of inflammatory conditions.

Modulation of HO-1, however, may not only serve as a therapeutic target in inflammatory disease, but also has therapeutic implications in organ transplantation. HO-1 has been demonstrated to play a protective role in several experimental transplantation models, in which elevated HO activity prevents the development of vascular lesions, intra-graft apoptosis, ischemia/reperfusion injury and significantly prolongs allograft survival (Soares et al., 1998; Hancock et al., 1998; Amersi et al., 1999; Immenschuh and Ramadori, 2000).

5. SUMMARY

Heme oxygenase-1 (HO-1) is the inducible isoform of the first and rate-limiting enzyme of heme degradation and is up-regulated by a host of stress stimuli. Activation of the HO-1 gene not only protects cells and tissues against oxidative damage, but also modulates the inflammatory immune response. Lipopolysaccharide (LPS) is a prototypical mediator of inflammation and is known to activate the phagocyte NAD(P)H oxidase. To further understand the regulatory role of HO-1 in mononuclear phagocytes during inflammation, the HO-1 gene regulation by LPS and by the NAD(P)H oxidase inhibitor 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF) was investigated in the monocytic cell line RAW264.7.

HO-1 gene expression was markedly induced by LPS and, unexpectedly, also by AEBSF in these cells. To determine the molecular mechanisms and signaling pathways of HO-1 gene expression by these compounds, reporter gene constructs with proximal HO-1 promoter gene sequences were examined in transiently transfected RAW264.7 cells. Up-regulation of HO-1 promoter activity by LPS was decreased by pharmacological NF-κB inhibitors and by overexpression of dominant negative mutants of NF-κB inducing kinase, inhibitor of NF-κB (IκB) kinase β and IκBα. The p38 MAPK inhibitor SB203580 and overexpressed dominant negative p38β decreased, whereas p38δ increased, LPS-dependent induction of HO-1 gene expression. Deletion and mutation analysis with transfected HO-1 promoter gene constructs indicated that a CRE/AP-1 site (-668/-654), but not an E-box motif (-47/-42), was involved in LPS-dependent HO-1 gene regulation.

AEBSF-dependent induction of endogenous HO-1 gene expression and promoter activity was abolished by treatment with chemical inhibitors of the phosphatidyl inositol 3-kinase/ protein kinase B (PKB) pathway and overexpression of dominant negative mutants of PKB. Accordingly, cotransfected constitutive active PKB markedly up-regulated HO-1 promoter activity. Inhibition of p38α and p38β prevented the induction of HO-1 gene expression by AEBSF. p38 was stimulated by AEBSF in a PKB-dependent manner as demonstrated by a luciferase assay with a Gal4-CHOP fusion protein. Deletion and mutation analysis indicated that both, the E-box and the CRE/AP-1 element, were essential for mediating the full response of HO-1 promoter activity to AEBSF. Cotransfection of the coactivator p300 and the basic helix-loop-helix transcription factor USF2 enhanced the AEBSF-dependent response of the HO-1 promoter activity.

Taken together, the data indicate that the NF-κB, PKB and p38 signaling pathways play an important regulatory role for the induction of HO-1 gene expression by LPS and the NAD(P)H oxidase inhibitor AEBSF in mononuclear phagocytes.

6. ZUSAMMENFASSUNG

Häm Oxygenase (HO)-1 ist die induzierbare Isoform des Schrittmacherenzyms des Hämabbaus und wird durch verschiedene Stressstimuli heraufreguliert. Die Induktion des HO-1 Gens schützt nicht nur Zellen und Gewebe vor oxidativem Stress, sondern ist auch an der Regulation der entzündlichen Immunantwort beteiligt. Lipopolysaccharid (LPS) ist ein prototypischer Auslöser von Entzündungen und aktiviert die monozytäre NAD(P)H Oxidase. Um die Rolle der HO-1 in mononukleären Phagozyten während Entzündungsreaktionen aufzuklären, wurde die Regulation des HO-1 Gens durch LPS und den NAD(P)H oxidase Inhibitor 4-(2-Aminoethyl) Benzensulfonyl Fluorid (AEBSF) in der Monozytenzelllinie RAW264.7 untersucht. In diesen Zellen wurde die Expression des HO-1 Gens nicht nur durch LPS, sondern überraschenderweise auch durch AEBSF induziert. Um die molekularen Mechanismen und die Signalwege der HO-1 Genexpression durch diese Substanzen zu bestimmen, wurden Reportergenkonstrukte mit der proximalen Promotorregion des Ratten HO-1 Gens in transient transfizierten RAW264.7 Zellen verwendet. Die LPS-abhängige Induktion der HO-1 Promotoraktivität wurde durch pharmakologische NF-κB Inhibitoren und durch Überexpression dominant negativer Mutanten der 'NF-κB inducing kinase', des 'Inhibitors von NF-κB kinase' sowie von 'IκBα' herunterreguliert. Der p38 MAPK Inhibitor SB203580 und die Überexpression einer dominant negativen Mutante von p38α führten zu einer Herabsetzung der LPS-abhängigen Induktion der HO-1 Genexpression. Durch Transfektionsstudien mit HO-1 gezielt mutierten Promotergenkonstrukten konnte gezeigt werden, dass ein CRE/AP-1 Element (-668/-654), jedoch nicht ein E-Box Motiv (-47/-42), an der durch LPS vermittelten HO-1 Genregulation beteiligt war. Die AEBSF-abhängige Induktion der endogenen HO-1 Genexpression und der HO-1 Promotoraktivität wurden durch Behandlung mit chemischen Inhibitoren des Phosphatidyl-inositol 3-Kinase/ Protein Kinase B (PKB) Signalwegs und durch Überexpression einer dominant negativen Mutante von PKB gehemmt. Kotransfektion einer konstitutiv aktiven Mutante der PKB führte zu einer deutlichen Heraufregulation der HO-1 Promotoraktivität. Durch spezifische Hemmung der p38α und p38β MAPK Isoformen wurde die Induktion der HO-1 Genexpression durch AEBSF verhindert. Mittels eines Reportergenansatzes mit einem Gal4-CHOP Fusionsprotein konnte gezeigt werden, dass die Aktivierung der p38 MAPK durch AEBSF downstream von PKB lokalisiert war. Anhand von Mutationanalyse wurde gezeigt, dass sowohl das CRE/AP-1 Element als auch das E-box Motiv an der Induktion der HO-1 Genexpression durch AEBSF beteiligt waren. Kotransfektionsexperimente mit dem Helix-Loop-Helix Transkriptionfaktor USF2 und dem Koaktivator p300 führten zu einer gesteigerten AEBSF-abhängigen HO-1

Promotoraktivität. Zusammenfassend zeigen diese Daten, dass die NF-κB-, PKB- und p38-Signalwege eine wichtige Rolle für die Induktion der HO-1 Genexpression durch LPS und den NAD(P)H oxidase Inhibitor AEBSF in Monozyten spielen.

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